EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS IN LONG-EVANS RATS

by

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ABSTRACT

EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS IN LONG-EVANS RATS

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Myelin basic proteins from the CNS of Long-Evans rats were studied in regard to their development with age and their ability to produce EAE, protect against EAE, and induce teratogenic effects.

Polyacrylamide disc electrophoresis studies demonstrated that two basic protein components were present in the CNS myelin of this rat strain. The larger component was comparable to the encephalitogenic protein found in most mammalian species, whereas the smaller component was comparable to the additional encephalitogenic protein found in certain rodent species.

Electrophoretic assays of basic protein extracts (pH 1.8) from the CNS myelin of rats at various stages of development (14 to 130 days) showed that both of the basic proteins were present at 14 days after birth.

Electrophoretic analyses of basic proteins extracted at pH 1.8 from the whole brains of maturing rats (1 to 130 days) suggested that the two basic proteins were present earlier than 14 days after birth. A faint electrophoretic band which appeared to correspond with the larger protein was detectable in the 1 day old brains. At 7 days after birth, two bands which were very close together in the 1 and 5 day old brains appeared to merge into a single band which corresponded with the smaller protein.

The different electrophoretic staining intensities of the two basic proteins in both the myelin and whole brain studies indicated that proportionally there was a greater increase in the smaller protein than in the larger protein during brain maturation. The whole brain studies also showed that the increase in the two basic proteins appeared to coincide with the reduction of other basic proteins in the maturing brain.

The investigations dealing with the total protein content of brain myelin in developing rats showed that there was an increase in protein (per cent dry weight) from 7.87 per cent at 10 days to 23.17 per cent at 130 days. The period of greatest accumulation of myelin protein appeared to occur from 10 to 30 days after birth.

The studies concerning the induction of EAE by sensitization with various amounts of rat, bovine, or guinea pig myelin basic proteins or whole guinea pig spinal cord in complete Freund's adjuvant indicated that this rat strain was not highly susceptible to EAE. Out of 54 experimental animals, only 4 demonstrated hind leg paralysis, and only 13 had EAE lesions in the CNS.

Guinea pig neuroantigens were the only agents which caused hind leg paralysis and they were more effective in producing EAE lesions than the rat basic proteins. Bovine basic protein did not result in the induction of any EAE lesions.

The experimental efforts to induce encephalitogenic tolerance in the offspring of rats were not successful. Maternal sensitization with 43.2 μg of rat myelin basic proteins in incomplete Freund's adjuvant on either the 4th day (preimplantation) or the 8th day (postimplantation) of pregnancy did not decrease the ability of the

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adult offspring to develop EAE lesions when they were sensitized with various amounts of rat basic proteins or whole guinea pig spinal cord in complete Freund's adjuvant.

The two encephalitogenic rat proteins did not produce teratogenic effects in young rats. Maternal sensitization with 131.2 μg of rat myelin basic proteins in incomplete Freund's adjuvant on either the 4th day or the 8th day of pregnancy did not cause any gross or internal abnormalities in 21 day old fetuses. There was also no histological evidence of CNS abnormalities in the fetuses.

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INTRODUCTION

The etiology and pathogenesis of the demyelinating diseases of the nervous system have been subjects of speculation and controversy since the latter part of the 19th century. Multiple sclerosis (MS), which is the most common form of the human demyelinating disorders, is very puzzling in terms of its pathogenesis and its etiology is unknown.

Multiple sclerosis is predominately a disease of young adults often affecting persons between the ages of 20-40 years. The incidence is very low prior to the first decade, rises to a peak in the third decade, and falls markedly in the fifth and sixth decades. There is a higher prevalence of the disease in women than in men and the peak incidence is reached at an earlier age in women.

A familial tendency for MS has long been recognized. However, twin studies have not demonstrated a genetic predisposition, and within affected families, there is no evidence of a common genetic pattern.

Several studies on the general pattern of distribution of MS in the population suggest that the disease is more frequent among higher socioeconomic groups and it is more common in regions with a temperate, rather than a tropical climate.

The disease process in MS is variable in duration. It may progress rapidly over a period of weeks to months in the acute form or it may persist for decades with alternating periods of exacerbation and remission in the chronic form.

The clinical symptoms of MS are extremely varied in nature, but the most frequent complaints encountered are numbness, cerebellar ataxia, paralysis, diplopia, blindness, incontinence, impotence, and mental dis-

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order. The variability of symptoms correlates with the pathological findings in which multiple lesions are shown to be scattered in a haphazard distribution throughout the white matter of the brain and spinal cord. Unique features of the disease which are difficult to explain, however, are the large lesions of intense demyelination, the plaques of incomplete myelin loss, and the poor correlation between the extent of involvement of the central nervous system (CNS) at the time of post-mortem examination and the patient's clinical condition before death.

During the many decades which have elapsed since the discovery of MS, only two reasonable hypotheses have been put forth as to the cause and mechanism of the disease. One hypothesis attributes them to an infective agent, as yet undiscovered, but most likely a virus. The other hypothesis attributes them to an antigenic substance, produced within the body or introduced from without, which produces an auto-allergy to the myelin of the brain and spinal cord.

At the present time, the idea that MS may represent an autoallergic response is the commonly held working hypothesis concerning the pathogenesis of this and related demyelinating disorders. In the future, it may be possible to demonstrate an interplay between the viral and auto-allergic mechanisms in demyelinating diseases.

The concept of an immunoallergic basis for MS emerged in the late 19th century. It grew out of the observations that some patients developed encephalomyelitis and paralysis after being inoculated with Pasteur's rabies vaccine. Since the encephalitogenic activity of the vaccine was a property of the nervous tissue and not the killed rabies virus, the concept of an allergic encephalomyelitis was conceived. The histological similarities between post-inoculation encephalomyelitis

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and acute MS fostered the idea of an autoallergic pathogenesis for naturally occurring demyelinating diseases.

One experimental approach for studying MS has been the use of the laboratory disease, experimental allergic encephalomyelitis (EAE). EAE is an inflammatory and demyelinating disease of the CNS which is produced by sensitizing animals with antigenic material in the form of whole CNS tissue, purified myelin basic proteins, or peptides derived from myelin basic proteins. The sensitization technique usually consists of the incorporation of CNS antigens in a saline-in-oil emulsion containing killed mycobacteria. This emulsion is commonly referred to as "Freund's complete adjuvant".

Over the last several decades, extensive research has been directed toward the investigation of the pathological, chemical, and immunological aspects of EAE with the hope that an understanding of these processes would clarify the mechanisms underlying MS. Despite the wealth of information which has accumulated about EAE, the relationship between this experimental disease and MS still has not been adequately clarified. The data which have been reported concerning the similarities and the dissimilarities between these two diseases have been reviewed by Levine, 1971; Nilsson, 1972; Paterson, 1972; Kies, 1973; and Reynolds, 1974.

In the present investigation, myelin basic proteins from the CNS of Long-Evans rats have been studied in regard to their development with age and their ability to produce EAE, protect against EAE, and induce teratogenic effects.

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REVIEW OF THE LITERATURE

Experimental allergic encephalomyelitis has been the subject of a symposium (Kies and Alvord, 1959a) and several review papers (Kolb, 1950; Cavanagh, 1956; Condie and Good, 1959; Paterson, 1959, 1966, 1968, 1969, 1971; Waksman, 1959a; Adams et al., 1965; Adams and Leibowitz, 1969; Alvord, 1970, 1972; Gerstl, 1972; Kies, 1973).

The following review presents historical information about EAE and includes reports on current research which is being done in this field.

A. Historical Background of EAE.

Experimental allergic encephalomyelitis is the oldest and most widely studied experimental model of an autoallergic disease. Historically, the concept that injections of nervous tissue may cause allergic reactions in normal nervous tissue emerged in the 1880's and 1890's following attempts to explain the neurological complications (encephalomyelitis, demyelination, paralysis) that occurred in some people after inoculations with Pasteur's rabies vaccine which was prepared from dried rabbit spinal cord containing attenuated rabies virus. The continued occurrence of neurological complications despite the use of phenol-treated spinal cord and brain containing killed rabies virus suggested that the encephalitogenic and paralytogenic activities of the rabies vaccine resided in the nervous tissue rather than the dead virus.

In the late 1890's, the first attempts were undertaken to produce experimental neurological lesions and paralysis in animals by the injection of normal nervous tissue. The results of these early investigations were generally inconclusive because of the irregular appearance of neurological symptoms. These early studies have been reviewed by

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Kolb (1950).

During the late 1920's and the early 1930's, it was demonstrated that mammalian brain tissue has organ-specific antigenic activity which may induce the production of antibrain antibodies in animals (Witebsky and Steinfeld, 1928; Lewis, 1933, 1934). In addition, at this time, the first convincing evidence for the encephalitogenic and paralytogenic activity of brain tissue was shown by Rivers and his associates (1933, 1935). An acute disseminated encephalomyelitis accompanied by paralysis was observed in monkeys that had received repeated injections of rabbit brain extracts over a period of several months. The pathological changes in the monkeys resembled those seen in humans after injections of rabies vaccine. Schwentker and Rivers (1934) also induced similar neurological complications in rabbits following repeated injections of rabbit brain extracts. These studies showed that acute disseminated encephalomyelitis could be produced in experimental animals after multiple injections of either homologous or heterologous brain tissue.

The application of Freund's immunological adjuvant to the study of tissue sensitization during the 1940's resulted in the development of the accelerated form of disseminated encephalomyelitis which is now known as EAE. At this time, it was shown that encephalomyelitis could be induced in guinea pigs (Freund et al., 1947), rabbits (Morrison, 1947), and monkeys (Kabat et al., 1946, 1947; Morgan, 1946, 1947) within a few weeks by a single or a few injections of CNS tissue emulsified in Freund's adjuvant. These findings firmly established EAE as a rapidly reproducible laboratory model for investigating immunoallergic reactions in the nervous system.

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B. Induction of EAE.

Experimental allergic encephalomyelitis has been produced in many mammalian and avian species by the injection of CNS myelin antigens combined with Freund's complete adjuvant. Susceptible species include monkeys (Kabat et al., 1946, 1947; Morgan, 1946, 1947; Jackson et al., 1972; Behan et al., 1973; Rauch et al., 1973), sheep (Innes, 1951), goats (Lumsden, 1949; Innes, 1951), pigs (Fog and Bardram, 1953), dogs (Thomas et al., 1950; Saragea et al., 1965; Hughes et al., 1966; Maros et al., 1966), cats (Paterson and Brand, 1957; Paterson, 1959), rabbits (Morrison, 1947; Waksman and Morrison, 1951; Prineas et al., 1969; Dona et al., 1971; Shapira et al., 1971; Bergstrand, 1973), guinea pigs (Freund et al., 1947, 1950; Alvord, 1948, 1949; Alvord and Kies, 1959; Alvord et al., 1959; Lampert and Kies, 1967; Hoffman et al., 1973; Driscoll et al., 1974; Raine et al., 1974), hamsters (Tal et al., 1958; Paterson et al., 1974), rats (Lipton and Freund, 1952, 1953; Paterson, 1958; Levine and Wenk, 1961; Kosunen et al., 1963; Bubis and Luse, 1964a; Paterson et al., 1970; McFarlin et al., 1973; Levine, 1974; Swanborg et al., 1974), mice (Olitsky and Yager, 1949a, 1949b; Olitsky et al., 1950; Lee and Olitsky, 1955; Levine and Sowinski, 1973; Boehme et al., 1974), chickens (Ranzenhofer et al., 1958; Siller, 1960; Lipton and Steigman, 1961; Blaw et al., 1967; Wick, 1973), and pigeons (Anderson and Vogel, 1961).

The antigens which induce EAE are present in the myelinated CNS tissue of humans, as well as all of the mammalian and avian species which have been studied so far. The encephalitogenic activity of nervous tissue in the lower, cold-blooded vertebrates has been tested in different mammalian species with varying results. Kabat, Wolf, and

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Bezer (1948) found that whole brain tissue from amphibian (frog) and fish (carp) species failed to induce EAE in monkeys. Paterson (1957) found that mixtures of whole brain and spinal cord tissue from turtles, snakes, and frogs were ineffective in producing EAE in Hartley guinea pigs, which are highly susceptible to the encephalitogenic activity of mammalian CNS tissue. In another study, Levine and Wenk (1963) showed that whole spinal cord tissue from turtles and frogs was capable of inducing EAE in Wistar rats, but that whole spinal cord tissue of the carp was totally ineffective. The results of the latter two studies have been recently confirmed by Martenson and his associates (1972a).

The antigenic components responsible for encephalitogenic activity are organ-specific, rather than species-specific. Either homologous or heterologous myelinated CNS tissue will induce EAE. In addition, Kabat, Wolf, and Bezer (1949) showed an animal's own (autologous) brain tissue is capable of producing EAE. In their experiment, monkeys were lobectomized and the excised brain tissue was stored over dry ice. After the animals had recovered from the operation, each monkey was injected with its own brain tissue emulsified in Freund's complete adjuvant.

In the years following this work, EAE has been induced in many specific inbred strains of laboratory animals after sensitization with isogeneic CNS tissue plus adjuvant. Paterson (1968) has pointed out that from an immunobiological point of view, sensitization with isogeneic CNS tissue would be equal to sensitization with autologous CNS tissue, if it is assumed that animals in an inbred strain do not have histogeneic differences. Thus, it would appear that the antigenic constituents in living CNS tissue which are responsible for EAE can act as autoantigens.

Various routes of inoculation have been used to produce EAE in susceptible species, but the most effective method of sensitization is by

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the intracutaneous and/or foot pad routes (Paterson, 1959, 1971; Good, 1963; Tilney, 1970). These two routes appear to be advantageous for the distribution of antigens via the lymphatics to the draining lymph nodes, a site which plays a crucial role in the production of the immune response associated with EAE.

As has been noted previously, the consistent production of EAE requires not only the antigens in myelinated CNS tissue, but also Freund's complete adjuvant which contains killed mycobacteria, mineral oil, and an emulsifying agent. The exact mechanism of action by which this adjuvant potentiates immunological responses is not yet known. The theories regarding its mode of action have been discussed by Burnet (1972) and Neifeld, Pierce, and Ohanian (1972). In general, the proposed theories may be divided into three main categories: (1) a slow release of antigen from the inoculation site resulting in prolonged, continual stimulation of the reticuloendothelial system (Herdegen et al., 1947; Herbert, 1966; White, 1967), (2) antibody formation by the granuloma at the inoculation site (Askonas and Humphrey, 1955; Hennessen, 1967; French et al., 1970), and (3) rapid systemic dispersion of the antigen (Freund, 1956; Humphrey and White, 1970).

Studies concerning the technical aspects of the usage of Freund's complete adjuvant have shown that the proportions of oil and emulsifying agent, and the proportions of CNS antigens and adjuvant are significant factors in the optimal production of EAE (Lee and Schneider, 1962; Shaw et al., 1962a).

Certain strains of rats which are highly susceptible to EAE require only oil and not killed mycobacteria in the injected adjuvant (Levine and Wenk, 1961; Paterson and Bell, 1962; Paterson, 1966), but these strains develop more severe EAE when the inoculum contains mycobacteria

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(Levine and Wenk, 1965a). All of the other species and strains studied to date require killed mycobacteria for the induction of EAE after a single injection of inoculum (Alvord, 1972).

Various Gram-positive and Gram-negative bacteria, as well as lipopolysaccharide fractions extracted from mycobacteria or Gram-negative organisms can be substituted for mycobacteria in the adjuvant, but they have not been found to be as effective as the whole mycobacterial cells on a dry weight basis (Lipton, 1959; Shaw et al., 1964). The factor which is effective in the adjuvant appears to be a complex of lipids with a variety of polysaccharides and amino acids. This complex affects mononuclear cells in many ways, but little is understood about its immunological effects (Farthing, 1961; Alvord, 1972).

Pertussis vaccine has adjuvant-like properties and it can be substituted for Freund's complete adjuvant in the production of EAE in mice (Lee and Olitsky, 1955) and guinea pigs (Wiener et al., 1959). In rats (Levine, 1974) and monkeys (Behan et al., 1973), pertussis vaccine produces a hyperacute form of EAE, and in the former species, the vaccine does not have to be incorporated into the antigenic CNS inoculum to cause its effects (Levine and Wenk, 1956b, 1966).

It is not clearly understood how the pertussis vaccine acts to potentiate and augment the responses to CNS tissue sensitization. It is known that the vaccine has the capacity to enhance immunological reactivity of the immediate or anaphylactic type associated with the release of histamine (Malkiel and Hargis, 1952), and it also causes a marked rise in the number of circulating lymphocytes (Morse, 1965). Thus, the adjuvant properties of the vaccine might result in an increased amount of lymphocytes sensitized to CNS tissue or an increased production of antibodies.

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C. Clinical and Pathological Aspects of EAE.

The extent to which the clinical and pathological signs of EAE develop during the course of the disease is dependent on the type and amount of antigens and adjuvant used for sensitization, the route of sensitization, and the specific strains and species of animals sensitized.

The clinical manifestations of EAE which are common in most animals are weight loss, weakness, tremors, fecal impaction, urinary incontinence, ataxia, impaired righting reflexes, and paresis or paralysis of one or both hind limbs (Cavanagh, 1956; Paterson, 1968; Levine, 1971).

The clinical symptoms generally occur two to three weeks after sensitization, although there may be some variation in the latent period. In most instances, if the animals survive the acute paralytic phase of the disease, they will show an eventual recovery. However, in some species, such as monkeys (Cavanagh, 1956; Paterson, 1959; Wolf, 1963), dogs (Thomas et al., 1950; Maros et al., 1966), and cats (Paterson and Brand, 1957) the clinical picture is often more complex and varied. The neurological symptoms may include nystagmus, anisocoria, strabismus, ptosis of the eyelids, blindness, facial weakness, convulsions, and fulminating quadriplegia. In addition, EAE in these animals may follow a remitting clinical course, with dramatic remissions and exacerbations.

The clinical features of EAE are related to the number, size, and location of the pathological lesions. Waksman (1959a) noted that the correlation between the clinical and pathological findings is excellent in monkeys, fair in rabbits and rats, and poor in guinea pigs and mice.

The characteristic pathological feature of EAE lesions is a vascular and perivascular inflammation of the brain and spinal cord which is accompanied by a variable degree of perivascular myelin damage

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(Cavanagh, 1956; Paterson, 1959, 1971; Waksman, 1959a; Alvord, 1970). The lesions are disseminated widely throughout the gray and white matter of the CNS, but they are most often found in the white matter and in areas near the ventricular system. Small, adjacent perivascular lesions may become confluent giving rise to large lesions which can be seen grossly. Lesions of this type are seen in monkeys (Rauch et al., 1973), dogs (Maros et al., 1966), and cats (Paterson and Brand, 1957).

The distribution of the lesions varies in different animal species. In monkeys (Cavanagh, 1956; Rauch et al., 1973), the optic nerves, cerebrum, brain stem, and cerebellum are mainly affected; in rabbits (Morrison, 1947; Waksman and Adams, 1962) and rats (Levine and Wenk, 1961; Levine, 1971), the spinal cord and lower brain stem are principally affected; and in guinea pigs (Waksman and Adams, 1962) and mice (Olitsky and Yager, 1949b; Levine and Sowinski, 1973), both the brain and spinal cord are involved.

The inflammatory lesions of EAE involve small blood vessels, notably, small veins or venules. The inflammatory cells accumulate within the vessel walls and extend beyond, forming one to several concentric layers of cells in the so-called perivascular space.

The cellular infiltrates consist predominantly of mononuclear cells - lymphocytes, monocytes, macrophages, and with time, some plasma cells. Polymorphonuclear cells are rarely present, except in severe cases of EAE, in which hemorrhagic and necrotic changes occur in the walls of the blood vessels and the surrounding nervous tissue. Lesions of this type are found in monkeys (Rauch et al., 1973), dogs (Thomas et al., 1950), and cats (Paterson and Brand, 1957) and they can be produced regularly in highly susceptible strains of rats by enhancing the intensity of immunization with pertussis vaccine. In rats with this hyperacute form of EAE, the clinical and pathological signs appear early

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(7 or 8 days after inoculation) and the mortality rate is very high (Levine and Wenk, 1965b; Levine et al., 1965; Levine, 1974).

An important element of the EAE lesions is the damage to the myelin sheaths around the nerve fibers going through or near the sites of vascular inflammation. Although areas of vasculitis may be observed without significant perivascular demyelination, the loss of myelin is not seen in the absence of associated vascular inflammation. Myelin damage occurs along with or follows after the vasculitis, which suggests that demyelination is secondary to vascular injury.

The degree of demyelination varies in different species of animals. Extensive myelin destruction is observed in monkeys (Rauch et al., 1973) and dogs (Maros et al., 1966). In contrast, only limited demyelination, which is present in the immediate area of perivascular inflammation, is seen in rabbits (Prineas et al., 1969), guinea pigs (Raine et al., 1974), rats (Levine, 1974), and mice (Lee and Olitsky, 1955).

The present view of the pathogenesis of EAE is that damage to the CNS tissue is brought about by mononuclear cells specifically sensitized to the myelin antigen. Evidence in support of this view has come from immunological studies which have shown the disease can be passively transferred with lymph node cells from sensitized donors (Paterson, 1960; Stone, 1961; Astrom and Waksman, 1962; Lee et al., 1965; Falk et al., 1968), but not with immune serum (Chase, 1959; Behan et al., 1973). Also, the severity of the disease does not correlate with the levels of serum antibody to the myelin antigen (Lisak et al., 1969; Driscoll et al., 1974).

An early event in the pathogenesis of EAE occurs during the first week after sensitization with the myelin antigen and adjuvant. At this

time, the local lymph nodes produce a large number of immunologically active cells which are directed against the myelin antigen (Kosunen et al., 1963; Lamoureux et al., 1968). These lymph node cells include specifically sensitized lymphocytes (Rauch and Raffel, 1964a; 1965) and other cells, including macrophages and plasma cells (Ridley, 1963), which are carried to the thoracic duct and the blood stream (Wenk et al., 1967). There are differing opinions about the length of time these cells circulate (Alvord, 1970), but some of them can be identified as DNA-, RNA-, and protein synthesizing cells which undergo mitosis and enlarge into "blast" cells after being stimulated by myelin antigen (Dowling and Cook, 1968).

Another early event in the pathogenesis of EAE is an increase in the vascular permeability of the CNS. This has been demonstrated by intravenous injections of Trypan blue (Barlow, 1956) and Thorotrast (colloidal thorium dioxide) (Lampert and Carpenter, 1965; Lampert, 1967); by autoradiography with labelled albumin (Vulpe et al., 1960) and labelled gamma globulins (Cutler et al., 1967); by electron microscopy (Luse and McDougal, 1960; Bubis and Luse, 1964a, 1964b; Levine et al., 1965); and by fluorescent microscopy (Oldstone and Dixon, 1968; Simon and Anzil, 1974).

Some of the foregoing studies have indicated that the permeability of the CNS blood vessels increases as early as 7 days after encephalitogenic challenge and this precedes the appearance of inflammatory mononuclear cells in the CNS (Vulpe et al., 1960; Oldstone and Dixon, 1968; Simon and Anzil, 1974). The factors responsible for this change in vascular permeability have not been clearly elucidated. Cytotoxic factors (Field, 1961), humoral antibodies (Oldstone and Dixon, 1968), antigen-antibody complexes (Alvord, 1970), and pharmacologically active

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substances (Simon and Anzil, 1974) have been suggested as possible agents.

Autoradiographic (Kosunen et al., 1963) and electron microscopic studies (Luse and McDougal, 1960; Bubis and Luse, 1964a, 1964b; Lampert, 1965, 1966, 1967; Lampert and Carpenter, 1965; Field and Raine, 1966; Lampert and Kies, 1967; Prineas et al., 1969; Raine et al., 1974) have demonstrated that between 10 to 20 days after encephalitogenic challenge, sensitized mononuclear cells pass between the endothelial cells and through the basement membrane of the small CNS blood vessels to infiltrate the adjacent parenchyma and damage the oligodendroglial cells and myelin sheaths.

Bubis and Luse (1964a) and Raine and his associates (1974) found that fewer oligodendroglial cells are present in the affected areas of the CNS than in normal areas. In addition, these cells have an abnormal appearance because of swollen organelles and disrupted cytoplasmic membranes. Lampert (1965, 1967) observed that these abnormal oligodendroglial cells are engulfed and phagocytosed by mononuclear cells, and in some places, they are obviously surrounded by the mononuclear cells prior to any visible cellular alteration.

Two distinct patterns of demyelination have been recognized in the presence of the mononuclear cells associated with EAE (Bubis and Luse, 1964a; Lampert, 1965, 1967; Lampert and Kies, 1967; Raine et al., 1974). One mechanism consists of a focal vesicular disintegration and extracellular lysis of the myelin lamellae in contact with the mononuclear cells. This form of myelin destruction is often preceded by a separation of the myelin lamellae. The other type of demyelination begins with an invasion of apparently normal myelin sheaths by the mononuclear cells which insinuate themselves between the myelin lamellae and strip the lamellae off of the axons. The displaced myelin fragments are engulfed

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by the cytoplasm of the mononuclear cells and later transformed into globoid lipid bodies. Macrophages containing these lipid bodies are conspicuous in advanced lesions.

In later stages of EAE and in the recovering animals, proliferative astrocytic responses occur. Increased numbers of mitochondria, ribosomes, and filaments are found in the astrocytes, and a spotty gliosis is formed by an interlacing of the astrocytic processes which are densely packed with fibrils (Luse and McDougal, 1960; Bubis and Luse, 1964a; Lampert, 1967; Raine et al., 1974).

Remyelination of the CNS axons occurs in small laboratory rodents (Luse and McDougal, 1960; Bubis and Luse, 1964a; Lampert, 1965, 1967; Prineas et al., 1969; Raine et al., 1974). Oligodendroglial cells, presumably derived from the surviving cells at the edge of the EAE lesions, proliferate and their processes spiral around the axons. The plasma membranes of these processes fuse and form myelin lamellae in a manner similar to that seen in the CNS during primary myelinogenesis (Luse, 1956, 1959).

D. Characterization of the CNS Encephalitogenic Antigen.

One of the most difficult and complex problems in the field of EAE has been the identification, purification, and characterization of the antigenic constituent which is responsible for the encephalitogenic activity of the CNS tissue.

An early assumption that myelin might be encephalitogenic was based on the observations that CNS white matter was more effective for the induction of EAE than gray matter (Kabat et al., 1947; Morgan, 1947; Alvord, 1949), and that encephalitogenic activity was not present in unmyelinated fetal CNS tissue (Kabat et al., 1947). Subsequently, it

was reported that white matter phospholipids (Alvord, 1948), lipids (Lumsden, 1949; Waksman and Morrison, 1951), and proteolipids (Olitsky and Tal, 1952; Goldstein et al., 1953; Waksman et al., 1954) had a low level of encephalitogenic activity.

The first report on an encephalitogenic protein which had been subjected to quantitative bioassay was that of Roboz, Henderson, and Kies (1958), who isolated a collagen-like protein from bovine spinal cord. This protein had a higher specific activity than any other encephalitogenic substance isolated at the time. In the following year, Kies and Alvord (1959b) and Roboz and Henderson (1959) extracted other encephalitogenic protein fractions from defatted guinea pig brain and bovine spinal cord, respectively. These protein fractions differed from the collagen-like protein in that they were obtained by acidic extraction methods. By 1961, Kies, Murphy, and Alvord had developed an extraction technique which produced a basic protein preparation from guinea pig brain with a high specific encephalitogenic activity.

It was difficult to prove that this basic protein was a component of myelin until a purified myelin fraction could be separated from whole CNS tissue. In 1962, Laatsch and his collaborators obtained a purified myelin fraction from guinea pig brain by ultracentrifugation and isolated the highly encephalitogenic basic protein from it. Rauch and Raffel (1964b) confirmed the localization of the basic protein in the myelin sheath of human, bovine, and guinea pig spinal cord sections by the technique of immunofluorescent microscopy. Although later studies also showed that this protein is a structural component of the myelin sheath, its exact position within the sheath (intraperiod line, major dense line, or both) has still not been definitely clarified (Kornguth and Anderson, 1965; Dickinson et al., 1970; Adams et al., 1971; Graham et al., 1974).

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Throughout the years, the encephalitogenic basic protein has been extracted from the whole CNS tissue, white matter, and myelin of several mammalian and submammalian species (Einstein et al., 1962; Kibler et al., 1964; Caspary and Field, 1965; Kies, 1965; Carnegie and Lumsden, 1966; Nakao et al., 1966a, 1966b; Eng et al., 1968; Carnegie, 1969a; Eylar et al., 1969; Martenson and Gaitonde, 1969a, 1969b; Martenson et al., 1971a, 1972a; Deibler et al., 1972; Jackson et al., 1972; Eylar et al., 1974; Swanborg et al., 1974). Although several extraction and fractionation methods have been employed by researchers, the essential step consists of the extraction of defatted (by chloroform-methanol or acetone) CNS material with aqueous solutions at an acid pH. The extract of basic protein which is obtained is virtually as encephalitogenic as the basic protein purified by gel filtration or ion-exchange chromatography.

Initial disagreements among investigators concerning the composition and molecular size of the basic protein were resolved by the discovery that an acid proteinase in the CNS tissue can degrade this protein into smaller fragments, depending on the type of solvents used for defatting the CNS tissue and what level of pH is used for the acid extraction (Nakao et al., 1966a, 1966b; Carnegie et al., 1967; Einstein et al., 1969; Hashim and Eylar, 1969a).

The technique commonly employed for examining the homogeneity of the basic protein preparations has been electrophoresis at pH 4.3 in 15 percent polyacrylamide gels. With this technique, the purified basic protein of most mammalian species migrates as a single protein component.

Two basic protein components have been demonstrated, however, in the CNS myelin of rodents in the suborder Myomorpha - rat (Eng et al., 1968; Martenson et al., 1969, 1970a, 1970b, 1971a, 1971b; Martenson and Gaitonde, 1969a, 1969b; Agrawal et al., 1970; Gaitonde and Martenson,

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1970; Kauttu, 1970; Kies, 1970; Mehl and Halaris, 1970; Wood and King, 1971), mouse (Greenfield et al., 1971; Martenson et al., 1971a; Morell et al., 1972), and hamster (Martenson et al., 1971a) and the suborder Sciuromorpha - squirrel, prairie dog, and woodchuck (Martenson et al., 1971a). In these rodents, the larger protein, which has a slower electrophoretic mobility, is comparable to the single encephalitogenic myelin basic protein of the other mammalian species. The smaller basic protein has a faster cathodic mobility.

Martenson and his co-workers (1971a) found that the additional smaller protein is absent from the CNS myelin of two rodents, the chinchilla and guinea pig, which belong to the suborder Hystricomorpha. They suggested that the genetic changes leading to the additional smaller basic protein (gene duplication followed by partial deletion) probably occurred after the rodent line leading to the suborder Hystricomorpha had been established and before the divergence of the remaining rodent line into the two suborders, Myomorpha and Sciuromorpha.

Studies regarding the myelin basic proteins of submammalian species have shown that the single basic protein present in the chicken (Mehl and Halaris, 1970; Martenson et al., 1972a), turtle (Martenson et al., 1972a), and frog (Martenson et al., 1972a) displays an electrophoretic mobility which is very similar or even identical with the myelin basic protein of most mammalian species.

The myelin basic proteins of the spiny dog-fish (Agrawal et al., 1971) and carp (Mehl and Halaris, 1970) have been characterized electrophoretically and found to be different from those of the mammalian species. Martenson and his associates (1972a) reported that the shark myelin basic protein has a major component and two minor components, whereas the carp myelin basic protein has two major components and

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several minor ones. The major shark component has a fairly fast electrophoretic mobility which is intermediate between the large and small rat basic proteins, while the two major carp components have a faster mobility than any of the basic proteins which have been examined to date. The authors suggested that the minor components in the shark and carp basic proteins may be breakdown products resulting from rapid autolytic changes which occur in the CNS tissue from cold-blooded species.

During the past decade, the physical, chemical, and biological properties of the myelin basic proteins have been studied in many laboratories in order to identify and localize specific determinants with encephalitogenic capacities.

The molecular weights of the myelin basic proteins from several mammalian and submammalian species have been found to be quite similar, approximately 18,400-18,500 (Eylar, 1970, 1972, 1973; Carnegie, 1971; Kies et al., 1972; Martenson et al., 1972a, 1972b; Reynolds and Green, 1973). Two exceptions are the smaller rat protein and the carp protein which have been reported to have molecular weights of approximately 14,400 (Kies et al., 1972).

An important characteristic of the basic protein molecule is its unfolded, open conformation which was first shown by viscosity studies with bovine and human basic proteins (Eylar and Thompson, 1969; Hashim and Eylar, 1969b; Palmer and Dawson, 1969; Chao and Einstein, 1970a; Oshiro and Eylar, 1970). Further evidence for this type of molecular conformation came from observations that the basic protein is very susceptible to small amounts of proteolytic enzymes (Hashim and Eylar, 1969a) and that it can be treated with 8 M urea or heated at 95°C for

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one hour without any lost of EAE-inducing activity (Eylar and Thompson, 1969).

The unfolded conformation of the basic protein suggested that its encephalitogenic determinants might be limited to short segments of the polypeptide chain, like the antigenic sites of unfolded synthetic polypeptides. Thus, information concerning the amino acid sequences in the basic protein molecule assumed major significance.

The complete amino acid sequences of the human (170 residues) (Carnegie, 1971), chimpanzee (171 residues) (Westall et al., 1975), bovine (169 residues) (Eylar et al., 1971; Brostoff et al., 1974), and smaller rat (130 residues) (Dunkley and Carnegie, 1974) basic proteins have been determined by the Edman degradation procedure using isolated tryptic and peptic digests. Partial amino acid sequences for other mammalian and submammalian basic proteins have been determined by comparing analyses of derived peptides obtained from peptide mapping (Kibler et al., 1969; Martenson et al., 1971c, 1972b, 1975b; Shapira et al., 1971; Brostoff and Eylar, 1972; Eylar, 1972, 1973; McFarlin et al., 1973; Eylar et al., 1974; Bernard and Carnegie, 1975; Martenson and Deibler, 1975).

The clarification of the amino acid sequences of the bovine and human basic proteins resulted in the discovery of some unusual features which are not often found in proteins, and which may be related to the unique role of the basic protein in the myelin sheath. First, a methylated arginine residue is present near the middle of the protein molecule. It is the only arginine residue which is methylated and it appears in all of the mammalian basic proteins which have been examined, as well as in the chicken and turtle proteins (Brostoff and Eylar, 1971). The investigators suggested that since methylation makes the arginine

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residue more energetically acceptable for nonpolar conditions, it is possible that this residue could stabilize interactions within the protein molecule or with the lipid components of the myelin sheath.

A second unusual feature of the protein molecule is a proline-rich region which includes a triproline sequence located near the methylated arginine residue. Eylar (1972) pointed out that the proline residues impose conformational restrictions which could produce a U-shaped bend causing the protein molecule to assume an open double chain structure. This idea has been recently supported by data indicating that the protein is shaped like a prolate ellipsoid (Epand et al., 1974).

A third unique characteristic of the protein molecule is a single threonine residue which acts as the specific attachment site for the polypeptide, N-acetyl-glactosaminyl-transferase (Hagopian et al., 1971). Only the myelin basic protein serves as a natural acceptor for this polypeptide which is an enzyme (from submaxillary glands) responsible for the formation of a sugar-protein linkage in the biosynthesis of certain glycoproteins (Hagopian and Eylar, 1968, 1969). Since the basic protein contains no sugar when it is isolated, researchers have suggested that the sugar residues may be attached to the protein during some early stages of myelin formation and then later removed (Hagopian et al., 1971).

Biochemical studies on the characterization of the myelin basic proteins have stressed the isolation of peptides by various enzymatic (Eylar and Hashim, 1968; Kibler et al., 1969; Palmer and Dawson, 1969; Eylar et al., 1971, 1972a; Shapira et al., 1971; Martenson et al., 1972b; Brostoff et al., 1974) and chemical (Eylar and Hashim, 1969; Carnegie et al., 1970; Burnett and Eylar, 1971; Swanborg et al., 1974) techniques in order to identify and define the encephalitogenic determinants.

IN 1968, Eylar and Hashim discovered for the first time that a 14-

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residue peptic peptide (bovine) containing a single tryptophan residue was as encephalitogenic in guinea pigs as the original basic protein. Carnegie (1969b) isolated an identical tryptophan-containing peptide from human basic protein and confirmed its high encephalitogenic activity in guinea pigs.

In 1970, Eylar and his associates isolated a tryptophan-containing peptide (bovine) consisting of only 9 amino acids (residues 114-122) which was highly encephalitogenic in guinea pigs. Subsequently, a large number of peptides were synthesized by the Merrifield solid state technique in order to determine what amino acids in the 9-residue peptide were essential for the induction of EAE in guinea pigs (Eylar et al., 1970; Westall et al., 1971). The results showed that the nonapeptide is inactive if the COOH-terminal lysine or the NH_2-terminal phenylalanine is removed. Hence, the minimum length of the encephalitogenic tryptophan region was established as 9 residues. In addition, 3 amino acids were shown to be essential in the peptide - tryptophan, glutamine, and the terminal basic residue (lysine or arginine). Replacement of any of these amino acids resulted in the loss of major encephalitogenic activity.

Two other regions of the basic protein molecule, residues 1-22 (Carnegie, 1969a; Lennon et al., 1970; Barton et al., 1972b) and residues 44-89 (Kibler et al., 1969), have been reported to be encephalitogenic in guinea pigs, but they have weak activity in comparison with the tryptophan region (residues 114-122).

Although other regions of the basic protein molecule may have small traces of activity in the guinea pig, there is rather convincing evidence that the nonapeptide tryptophan region is the major encephalitogenic determinant in this species. The isolated (Eylar et al., 1970) or syn-

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thesized (Westall et al., 1971) peptide is approximately equal to the original basic protein on a molar basis in its capacity to induce EAE in guinea pigs. The basic protein is essentially inactive when the single tryptophan residue is blocked by a reaction with 2-hydroxy-5-nitrobenzyl (HNB) bromide (Eylar and Hashim, 1969; Chao and Einstein, 1970b; Swanborg, 1970; Swanborg et al., 1974). The basic proteins of many mammalian species (human, monkey, horse, bovine, sheep, dog, rabbit, guinea pig, and larger rat component) have the same encephalitogenic activity in guinea pigs and the same amino acid sequence in the tryptophan region, except the rat (larger component) and human, which have an acceptable replacement of lysine with arginine (Eylar, 1971, 1972; Eylar et al., 1974).

The smaller rat basic protein and the chicken basic protein are both relatively nonencephalitogenic in guinea pigs, and both have modified amino acid sequences in the tryptophan region (Eylar, 1971; Martenson et al., 1972a, 1972b; Eylar et al., 1974). The smaller rat protein lacks a sequence of approximately 40 amino acid residues. The deletion begins just beyond the single tryptophan residue and results in a severely altered tryptophan region in which the glutamine residue is lost (Martenson et al., 1972b; Dunkley and Carnegie, 1974). In the tryptophan region of the chicken basic protein, the glutamine residue is also absent, being replaced with histidine (Eylar et al., 1974). It is believed that the loss of the critical glutamine residue in both of these basic proteins may be the primary reason for their relative lack of encephalitogenic activity in guniea pigs.

The tryptophan region of the basic protein is not the major encephalitogenic determinant in some of the other mammalian species which have been studied, and it appears that regions of the basic protein

responsible for EAE may vary from species to species.

In the rabbit, the tryptophan-containing peptide (residues 114-122) is highly encephalitogenic (Eylar et al., 1971), but other regions of the basic protein molecule also induce EAE in varying degrees. These regions have been localized by peptides numbered with the following amino acid sequences: 1-20 and 65-74 (Shapira et al., 1971), 44-68 (Bergstrand, 1973), 44-89 (Eylar and Brostoff, 1971; Kibler et al., 1972), 117-170 (Bergstrand and Berg, 1971), and 134-150 and 154-170 (Bergstrand, 1972).

In the monkey, the tryptophan region of the basic protein molecule is not encephalitogenic (Jackson et al., 1972). Eylar and his associates (1972a) tested a group of peptides to ascertain which regions of the protein molecule were responsible for EAE in the monkey. They found that a major encephalitogenic site for this species is located in a 53-residue peptic peptide in the C-terminal region of the molecule. They further defined this site by finding that a 37-residue peptic peptide (residues 134-170) is as active on a molar basis as the original basic protein. They observed that this latter peptide is at least 10-fold more active than two other peptides containing residues 1-43 and 44-89, respectively, which have mild encephalitogenic activity in the monkey.

In the rat, it appears that the tryptophan-containing peptide (residues 114-122) is not critical because the smaller rat basic protein, which lacks the last half of this sequence, is highly encephalitogenic in this species (Martenson et al., 1972a). In addition, studies with 2-hydroxy-5-nitrobenzyl (HNB) bromide, which blocks the tryptophan residue, have shown that this residue is not essential for the encephalitogenic activity of the bovine (Swanborg and Amesse, 1971), guinea pig, and larger rat (Swanborg et al., 1974) basic protein in

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this species.

In 1973, Dunkley and his co-workers isolated a peptide fragment from the smaller rat basic protein (residues 45-86) which was, on a molar basis, as encephalitogenic in rats as the intact protein.

In another study in 1973, McFarlin and his associates isolated peptides containing 45 residues (residues 44-89) from guinea pig, bovine, and the smaller rat basic proteins. When these peptides were tested for activity in rats, it was found that the guinea pig peptide was as highly encephalitogenic as the intact protein. The activity of the rat peptide was also comparable to that of the intact protein. However, the bovine peptide was inactive, in contrast to the intact protein which was mildly encephalitogenic. The investigators suggested that since the intact bovine basic protein was mildly active, this molecule might contain other encephalitogenic regions which were different from the 45 residue peptide they investigated.

Recently, Martenson and his co-workers (1975c) conducted further tests on regions of the bovine and guinea pig basic proteins which are encephalitogenic in rats. They found that the bovine protein has at least two mutually exclusive encephalitogenic regions. The more active region, which is located within the N-terminal half of the protein, includes the sequence Asp-Ser-Leu-Gly-Arg-Phe (residues 37-42). The less active region is located within the C-terminal half of the protein. It begins between residues 88 and 111 and ends before residue 153. This region contains the sequence Leu-Ser-Leu-Ser-Arg-Phe (residues 108-113), which is very similar to the first encephalitogenic determinant.

The studies by Martenson and his associates (1975c) showed that the high encephalitogenic activity of the guinea pig basic protein in rats could be fully recovered in the peptides containing residues 37-88 and

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43-88. This finding confirms the earlier report by McFarlin and his co-workers (1973) that a potent encephalitogenic determinant for rats exists in this region of the guinea pig protein. Martenson and his associates (1975c) also reported that there appears to be a weaker encephalitogenic determinant in the guinea pig peptide containing residues 89-169 since this peptide was found to have the same activity as the corresponding bovine peptide.

In summary, the foregoing investigations have shown that variations exist in regard to the peptide determinants responsible for the induction of EAE in different species. These variations are not well understood at the present time, but they may reflect genetic and immunologic specificities.

E. Development of Encephalitogenic Activity During CNS Maturation.

Kabat, Wolf, and Bezer (1947, 1948) conducted the earliest investigations on the appearance of encephalitogenic activity in the developing CNS of animals. In their studies, monkeys were used to assay the encephalitogenic activity of the brains and spinal cord of rabbits at various ages. According to their observations, the fetal rabbit brain was not encephalitogenic. Encephalitogenic activity did not appear in the brain until these animals were 12 days of age, but it was already present in the spinal cord of 3 day old animals.

In a later study, Waksman (1959b) reported that the spinal cords of rats, from birth to 4 days of age, were not capable of inducing EAE in adult animals of the same species. His findings indicated that encephalitogenic activity was present in the rat spinal cord at 7, 10, 14, and 21 days after birth, but this activity was weak and did not reach adult levels even at the postnatal age of 21 days.

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Svet-Moldavskij and his associates (1965) used guinea pigs to assay the development of encephalitogenic activity in the CNS of various animals. Their studies showed that in the mouse brain, encephalitogenic activity was absent up to and including the 12th day after birth. On the 16th day, the mouse brain was capable of inducing EAE. The brain of rats was not encephalitogenic by the 18th day of postnatal life, but it caused a weak encephalitogenic reaction by the 20th day after birth. The rabbit brain was encephalitogenic as early as the 2nd day after birth, but its encephalitogenic activity was much weaker than that of the adult brain. The brain of guinea pigs and of sheep was highly encephalitogenic even on the first day after birth. In addition, in all of the animals which were tested, the spinal cord was already encephalitogenic towards the end of fetal development.

After the discovery that the encephalitogenic activity of the CNS resides in the basic protein components of myelin, researchers began to investigate the distribution and quantity of these proteins during various stages of development.

In an early study, Einstein and Csejtey (1966) investigated the electrophoretic patterns of myelin proteins in the human brain beginning with 4 and 6 week premature infants. No definite electrophoretic band appeared to correspond with the encephalitogenic basic protein until the age of 4 years. There were no brain samples available, however, between the ages of 8 months and 4 years.

In the foregoing study, the brain protein preparations were also tested for encephalitogenic activity in guinea pigs. The preparation from a 10 week old infant was not encephalitogenic, but the preparation from an 8 month old infant was capable of inducing EAE in 1 out of 6 of the test animals. Encephalitogenic activity appeared to increase

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with brain maturation since the preparation from a 4 year old child was moderately encephalitogenic, whereas that from a 6 year old child was highly encephalitogenic.

In a subsequent investigation, Eng and his co-workers (1968) reported that an electrophoretic band which corresponded with the encephalitogenic basic protein was already present in the brain myelin of a human infant at 10 weeks of age. Their findings also indicated that the amount of the basic protein in myelin (dry weight) increased from 16 to 28 per cent during maturation from infancy (10 weeks) to adulthood.

In 1972, Savolainen and his collaborators studied the electrophoretic distribution of human myelin proteins in brain samples from 13 fetuses (11 to 19 gestational weeks), a 4 week premature infant, a 1 month old infant, and a 6 and 13 year old child. They reported the encephalitogenic basic protein could not be detected in the samples of brain myelin from the fetuses and the infants. It could be distinctly identified, however, in the brain myelin obtained from the 6 year old child, and its relative amount was greater in the myelin of the more mature 13 year old child.

Recently, Fishman and his associates (1975) conducted a comprehensive electrophoretic investigation of the protein changes in human brain myelin from birth to adulthood. They observed that the encephalitogenic basic protein was present in the brain myelin of newborn infants. They also noted that the amount of the basic protein increased from 8.5 per cent of the total myelin protein (dry weight) in the newborn to approximately 30 per cent in the adult. This study confirms the earlier work of Eng and his co-workers (1968).

Two electrophoretic studies on myelin proteins in the developing

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bovine brain have indicated that the encephalitogenic basic protein was already present in the brains of 30-65 cm crown-rump length fetal calves (Hegstrand and Kornguth, 1973) and 9 month old fetal calves (Fewster et al., 1974). In the former report, there was also evidence of an increase in the amount of basic protein during development from the fetal period to adulthood.

Einstein and her associates (1970) investigated the electrophoretic distribution of myelin proteins in the developing brain and spinal cord of rabbits. They reported that the earliest time at which the encephalitogenic basic protein could be dectected in either the brain or spinal cord was on the 9th day after birth. At this time, there was a higher concentration of the basic protein in the spinal cord than in the brain. This finding would appear to correlate with the fact that myelination occurs earlier in the former region than in the latter. These investigators also noted there was a general increase in the basic protein during maturation of the CNS.

Electrophoretic studies pertaining to the two encephalitogenic myelin basic proteins found in the brains of mice have indicated that these two proteins were already present as single electrophoretic bands at 15 (Matthieu et al., 1973), 10 (Greenfield et al., 1971), and 8 (Morell et al., 1972) days after birth. In addition, quantitative studies by Morell and his co-workers (1972) showed that the proportion of total myelin protein (dry weight) which was represented by the sum of the two basic proteins increased from about 18 to 30 per cent in animals from 8 to 300 days of age.

The brains and spinal cords of rats at various ages have been used to study the two encephalitogenic myelin basic proteins in this species. In an early investigation, Kornguth and his associates (1966) used

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fluorescent-labelled antibody to guinea pig myelin basic protein to demonstrate that an antigenic protein similar to the myelin basic protein was already present in the spinal cords of rats on the 21st day of gestation.

Eng and his collaborators (1968) reported that the two encephalitogenic basic proteins were present as single electrophoretic bands in myelin isolated from the brains and spinal cords of 16 day old postnatal rats. In 1971, Wood and King conducted a similar study on brain myelin from 16 day old postnatal rats and they found that the two encephalitogenic basic proteins were each split into two electrophoretic bands. This observation was probably the result of enzymatic degradation of the two proteins since more recent investigations have shown that each of the proteins could be clearly detected as a single electrophoretic band in the brain myelin of 14 day old postnatal rats (Agrawal el al., 1972; Druse et al., 1974).

Gaitonde and Martenson (1970) conducted a comprehensive electrophoretic study of the basic proteins in the brains of postnatal rats at 5, 10, 15, 21, 36, 63, and 82 days of age. Neither of the two encephalitogenic basic proteins were demonstrable in the brains of 5 day old rats. The smaller basic protein could be seen in the brains at 10 days after birth, but the larger basic protein could not be identified at this time. The researchers indicated that the presence of the latter protein might have been obscured by other electrophoretically slower moving components. By the 15th day after birth, however, both of the basic proteins could be distinctly identified as separate electrophoretic bands. In addition, quantitative studies by these same investigators showed that the two basic proteins together increased from 0.13 to 1.84 mg/g wet brain in animals at 15 and 55 days of age, respectively.

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In a later study, Adams and Osborne (1973) investigated the electrophoretic distribution of the protein components in brain myelin from postnatal rats between 5 and 60 days of age. They reported that at 5 days after birth neither of the two encephalitogenic basic proteins were detectable in the preparations they defined as 'early myelin'. At 7 days, an electrophoretic band corresponding with the larger basic protein was present, but it was not until 11-12 days after birth that the smaller basic protein also made its appearance and there was a changeover to adult type myelin. The investigators also noted that during the 12-60 day period examined there was a proportional decrease in the larger basic protein and a large relative increase in the smaller basic protein.

Recently, Zgorzalewicz and his associates (1974) were able to conduct a comprehensive electrophoretic study on the myelin proteins in three distinct regions of the CNS of rats ranging in age from 5 to 365 days (postnatal). They isolated separate myelin preparations from the forebrain, cerebellum, and spinal cord of animals as early as 15, 10, and 5 days after birth, respectively. The two encephalitogenic basic proteins could be observed as separate electrophoretic bands in each of these areas at the different stages of development which were examined. The researchers also reported that during maturation the ratio of the smaller basic protein to the larger basic protein increased in each of the three CNS areas which were studied.

In summary, the foregoing investigations have shown that there is an increase in encephalitogenic activity during CNS maturation which correlates with the development of the myelin basic protein(s).

F. Prevention, Suppression, and Therapy of EAE.

An arbitrary distinction is made between the terms prevention,

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suppression, and therapy of EAE: prevention consists of treatments given before the administration of the encephalitogenic antigen, suppression consists of treatments given after the administration of the encephalitogenic antigen, and therapy consists of treatment given after the onset of the clinical symptoms of the disease.

Throughout the years, a variety of procedures and pharmacological agents have been used for the prevention, suppression, or therapy of EAE. Generally, these techniques have been directed at depressing the development of the immune response or the development of the hypersensitive state. They have included the use of X-irradiation (Allegranza, 1959; Condie and Nicholas, 1962; Paterson and Beisaw, 1963; Scheinberg et al., 1967); thymectomy (Arnason et al., 1962; Janković et al., 1962; Janković and Išvaneski, 1963; Lennon and Byrd, 1973; Gonatas and Howard, 1974); specific antilymphocyte antisera (Waksman et al., 1961; Leibowitz et al., 1968a, 1968b; Brendel et al., 1969); non-specific stress reactions (Levine et al., 1962); and immunosuppressive drugs such as ACTH (Moyer et al., 1950; Gammon and Dilworth, 1953), cortisone (Kabat et al., 1952), melengestrol and hydrocortisone (Greig et al., 1970; Elliott et al., 1973a, 1973b), 6-mercaptopurine (Hoyer et al., 1962), methotrexate (Brandriss, 1963; Brandriss et al., 1965), and cyclophosphamide (Calne and Leibowitz, 1963; Paterson et al., 1967; Rosenthale et al., 1967; Paterson and Drobish, 1969; Paterson, 1971).

In order to avoid the systemic resistance-lowering effects of the foregoing techniques, researchers have attempted to modify the course of EAE by using more immunologically specific agents, such as whole CNS tissue, myelin basic proteins, or myelin basic peptides.

Ferraro and his co-workers (1949a, 1949b, 1950) were the first to

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observe that EAE could be partially prevented in guinea pigs by intramuscular injections of whole brain tissue (guinea pig or monkey) in incomplete Freund's adjuvant* prior to sensitization with guinea pig brain tissue plus complete Freund's adjuvant.

Later studies by Condie and his associates (1957, 1959) demonstrated there was a dramatic inhibition of EAE in rabbits subjected to repeated subcutaneous or intraperitoneal injections of rabbit spinal cord in saline prior to the injection of rabbit or bovine spinal cord with complete Freund's adjuvant.

In 1958, Paterson reported that rats given a single intraperitoneal injection of adult guinea pig spinal cord in saline, 1 or 9 days after birth, showed a striking decrease in the development of EAE when they were challenged at 8 to 10 weeks of age with guinea pig spinal cord plus complete Freund's adjuvant.

Waksman (1959b) reported similar results in rats pretreated neonatally with adult rat or rabbit spinal cord in saline. His study also showed the EAE could be inhibited in adult rats pretreated with repeated subcutaneous injections of rat or rabbit spinal cord in saline.

Kies and her collaborators (1960) and Shaw and his co-workers (1960, 1962b) reported partial prevention and suppression of EAE in guinea pigs by repeated injections (intracutaneous or intravenous) of encephalitogenic guinea pig brain proteins in incomplete Freund's adjuvant or saline before or at intervals after sensitization with guinea pig or bovine CNS tissue plus complete Freund's adjuvant.

Similar preventive and suppressive-type results were reported in

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* Incomplete Freund's adjuvant contains saline, mineral oil, and an emulsifying agent. It does not have the addition of killed mycobacteria which are present in complete Freund's adjuvant.

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later studies in which guinea pigs (Alvord et al., 1965; Cunningham and Field, 1965; Einstein et al., 1968; Lisak et al., 1970; Eylar, 1971; Swanborg, 1972; Teitelbaum et al., 1972), rats (Levine et al., 1970; Swanborg, 1973), and monkeys (Jackson et al., 1972) were injected intracutaneously with purified myelin basic proteins (homologous or heterologous) in incomplete Freund's adjuvant before or soon after sensitization with various encephalitogenic emulsions.

Einstein and her associates (1968) were the first to report that the clinical signs of EAE could be partially suppressed in guinea pigs by intracutaneous injections of high doses of non-encephalitogenic basic neural proteins (from bovine spinal cord and immature human brain) in incomplete Freund's adjuvant after encephalitogenic challenge. No suppressive effect was provided, however, by basic proteins (histones) of a non-neural origin.

According to a more recent study, pretreating guinea pigs with a purified non-encephalitogenic protein from the white matter of bovine spinal cord in incomplete Freund's adjuvant before encephalitogenic sensitization prevented all clinical and almost all histological evidence of EAE (MacPherson and Yo, 1973).

Eylar (1971, 1972) reported that the 2-hydroxy-5-nitrobenzyl (HNB) bromide derivative of human myelin basic protein, which is not encephalitogenic in guinea pigs, is effective in the prevention and suppression of EAE in this species. In addition, non-encephalitogenic peptides from human (Barton et al., 1972a) and bovine (Hashim and Schilling, 1973) myelin basic proteins have also been found to prevent EAE in guinea pigs.

Recently, Teitelbaum and her co-workers (1971, 1972, 1973) synthesized an amino acid copolymer (designated Cop 1) which is composed

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of alanine, glutaminic acid, lysine, and tyrosine and which has a molecular weight of 23,000. This basic copolymer is an efficient suppressor of the clinical and histological signs of EAE in guinea pigs and rabbits which have been sensitized with bovine or human myelin basic protein.

It was found that intravenous administration of the copolymer in saline was the most efficient route for effective suppression. The time and dosage of the injections were also very important. Repeated injections of 1 mg starting as late as 5 days after encephalitogenic sensitization were suppressive, whereas a single large dose of 5 mg given 72 hours after sensitization did not suppress EAE. The copolymer does not appear to be a non-specific immunosuppressant, since it does not affect the immune response towards various antigens, including the rejection of a skin graft.

One explanation for the suppressive action of the copolymer is that it may cause "tolerization" of the lymphoid cells sensitized to the basic protein or possible deviation of the immune response. An alternative explanation for its suppressive effect may lie in the basicity of the copolymer in regard to the net electrical charge or distribution of the charge on the molecule (Webb et al., 1973).

The prevention and suppression of EAE by myelin basic proteins and their derivatives have received considerable attention throughout the years, but there have been very few reports concerning therapy of the disease once its clinical symptoms have been established. This subject requires intensive future study because the possibility of a connection between EAE and MS has resulted in therapeutic trials of the human basic protein in patients with MS (Campbell et al., 1973).

Alvord and his associates (1965) were the first to report that

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after the onset of the clinical signs of EAE in guinea pigs, daily subcutaneous or intraperitoneal injections of homologous basic protein in saline could temporarily reverse the clinical signs and decrease the histological severity of the disease.

In 1972, Levine and his collaborators studied the therapeutic effects of rat, guinea pig, and monkey myelin basic proteins on established EAE in rats. One or more intravenous injections of 0.2 mg of basic protein in saline reduced the clinical and histological signs of the disease. This effect was shown in the histological lesions 2 or more days after the start of treatment and in the clinical improvement after 4 days. There was evidence of a certain degree of species specificity insofar as rat or guinea pig basic protein was more effective than monkey basic protein in treating EAE induced with rat spinal cord and complete Freund's adjuvant. However, monkey basic protein seemed to be a very efficient therapeutic agent when EAE was induced with monkey spinal cord and adjuvant.

Eylar and his co-workers (1972b) reported that the clinical signs of EAE in monkeys (induced with human basic protein in complete Freund's adjuvant) could be completely reversed by treatment with intramuscular injections of human basic protein or HNB-modified human protein (with a blocked tryptophan residue) in incomplete Freund's adjuvant.

In this study, the therapeutic injections were given within 24-48 hours after the appearance of the clinical symptoms. Improvement in these symptoms was noted within 24 hours. After the first injection, 2-3 mg of the protein was administered daily for at least 12 days of treatment. The clinical symptoms generally disappeared after 7 days of treatment.

The course of treatment greatly influenced the therapeutic effect.

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As long as the treatment exceeded a certain critical period (10-12 days), the monkeys showed no indication of a relapse. If the daily successive injections were stopped during the critical period, the clinical symptoms reappeared after 3-7 days. Permanent recovery could be achieved in these instances, however, if the daily injections were resumed.

The authors suggested that their success in treating EAE appeared to be due to a direct destructive action of the injected basic protein on the sensitized lymphocytes or stem cells of the central lymphoid tissue which mediate the disease.

Recently, Driscoll and his associates (1974) repeated the experimental procedures of Eylar and his collaborators (1972b) in treating guinea pigs with established clinical signs of EAE. Therapy was begun 24-48 hours after the onset of definite neurological symptoms and consisted of intramuscular injections of guinea pig basic protein in incomplete Freund's adjuvant for 10 days. Within 24 to 48 hours, the neurological symptoms disappeared and the guinea pigs regained their normal appearance and motor function.

The animals were sacrificed 4 weeks after the end of the treatment. The histological examinations did not reveal the inflammatory lesions commonly found in the brains and spinal cords of guinea pigs after sensitization with encephalitogenic basic protein plus complete Freund's adjuvant.

The researchers reported that the serum levels of antibody did not correlate with the severity of the disease or the success of the treatment. They concluded that a positive response to the treatment was a result of some change in the circulating lymphoid-sensitized cells.

G. Teratogenic Effects of CNS Tissue.

A few studies have indicated that CNS defects may be induced in

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embryos as a result of maternal immunization with brain tissue.

Gluecksohn-Waelsch (1957) was the first to report studies of this type concerning the induction of malformations of the CNS. She conducted two series of experiments. In the first, female mice of the Bagg albino strain were sensitized prior to breeding with intramuscular injections of brain tissue from Swiss Webster mice combined with adjuvants following the technique of Freund and McDermott (1942). Thirty-seven mice became pregnant and 321 embryos were examined on the 10th day after copulation. Twenty of these embryos had abnormalities of the CNS and 16 were dead and resorbed. These results were not significant, however, since abnormalities were also found in the uninjected controls. Thus, a second experiment was conducted using mice of the DBA/1 Glw strain, which was free of sporadic abnormalities of the CNS.

DBA/1 Glw females were injected with emulsions of brain or heart tissue combined with Freund's adjuvants at various time periods (4 days to 9 weeks) before breeding. Eight to 9 per cent of the embryos from females treated with brain tissue showed abnormalities of the CNS on the 10th day after copulation. These abnormalities consisted of suppression of nervous tissue differentiation in the area of the brain and the anterior spinal cord, microcephaly, and abnormalities in the closure of the neural folds. The embryos from females treated with heart tissue had no CNS defects, but one did have an abnormal heart.

The author suggested that the CNS abnormalities observed in her experiments might be due to the effect of maternal antibrain antibodies on the developing nervous system of the embryos.

In 1959, Barber, Afeman, and Willis used injections of homologous brain tissue plus complete Freund's adjuvant to sensitize female mice

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having normal eyes and pigmentation, as well as female mice having recessive factors for bilateral anophthalmia and albinism. The females were treated with intramuscular injections and were inbred (normals) and crossbred one week after the last injection. The subsequently conceived embryos were examined on the 13th day of gestation.

Eight-five embryos were obtained from 8 pregnant mice treated with brain tissue. All of the embryos from both the inbred normal and crossbred females had approximately the same degrees of retardation. The eyes were present and contained pigment, but they were retarded in development. The differentiation of the CNS appeared fairly normal except for the absence or retardation of the hypophysis and infundibulum. In some embryos, there was no indication of the initiation of development of Rathke's pouch or the infundibular process, whereas in others, the hypophysis was present but poorly differentiated. In addition, the axial structures were not well developed, the lower jaw and tongue were almost completely suppressed, and the liver and heart were poorly developed.

The authors concluded from their findings that maternal immunization with brain tissue appears to specifically retard the development of the hypophysis, and this in turn results in the lack of growth and development of other structures.

In a later study, Barber and her collaborators (1961) sensitized female mice with injections of homologous or heterologous (rabbit) brain tissue combined with complete Freund's adjuvant. They felt that in the earlier studies of this sort, anomalous embryonic development may have been seriously complicated by trauma and intra-uterine death. Hence, in these experiments, smaller doses of brain tissue were given over a longer period of time.

The treatment consisted of intramuscular injections given prior to

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breeding followed by a challenge dose on the first day of pregnancy. The embryos from the pregnant females were examined on the 14th and 19th day of gestation.

Eight females which had been sensitized with mouse brain emulsions produced 70 embryos of which 46 had defective lenses. Eleven females which had been sensitized with rabbit brain emulsions produced 97 embryos of which 60 had lens defects. No brain abnormalities were reported for any of the embryos in the two experimental groups.

The histological examinations of the embryonic lenses showed that by the 14th day of gestation, the primary fibers contained very fine vacuoles and droplets. By the 19th day of gestation, large fluid-filled vacuoles and cystoid degenerative changes were present in the center of the lenses. Secondary fibers which are formed at this stage of development appeared to be undamaged. No essential differences were observed in the effects of maternal immunization with homologous or heterologous brain tissue.

The authors suggested that the degenerative changes in the lenses of embryos from sensitized mothers might be the result of a non-specific antibody response. They stated that these changes closely resembled the cytological changes found in human embryonic lenses after maternal rubella.

Malformations of the CNS may also be induced in embryos as a result of maternal immunization with tissue-specific kidney antiserum which acts as a general teratogenic agent.

Brent, Averich, and Drapiewski (1961) were the first to report the deleterious effects of this teratogenic agent. In their study, 31 female rats were given a single intravenous injection of rabbit anti-rat-kidney antiserum on the 8th day of pregnancy. At the 21st day of gestation, the

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mothers were sacrificed and the embryos were examined.

Fourteen of the 31 litters obtained from the pregnant rats treated with kidney antiserum had severe malformations of the CNS as well as other systems. Four samples of the antiserum produced 100 per cent malformations if the dose was between 0.4 and 0.7 ml. Doses of 0.7-1.0 ml of the antiserum caused complete resorption of all of the embryos.

In a subsequent study, David and his associates (1963) administered a single intravenous injection of rabbit anti-rat-kidney antiserum to female Wistar rats on the 8th, 9th, 10th, and 11th day of pregnancy. The embryos were examined 17 to 20 days after copulation.

The authors reported that there were differences in the types and percentages of embryonic malformations as a result of treatment on the various days of pregnancy. Treatment on the 9th day of pregnancy produced the largest number (72 per cent) of malformed embryos. Exencephaly was the only CNS malformation found. Several embryos showed varying degrees of this anomaly after treatment on the 9th day of pregnancy, but there was only one example of it in an embryo after treatment on the 10th day of pregnancy.

In a similar study by Brent (1964), female Wistar rats received an intraperitoneal injection of rabbit anti-rat-kidney antiserum on the 7th, 8th, 9th, and 10th day of pregnancy. The embryos were examined on the 21st day of gestation.

Different arrays of embryonic malformations were produced, to some extent, as a result of treatment on the various days of pregnancy. Out of the 522 experimental embryos in this study, 234 had malformations. These malformations involved several different organs and systems, including the CNS. In regard to the CNS malformations, meningocele occurred in 1.2 per cent of the embryos after treatment on the 8th day of pregnancy;

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anencephaly in 2.4 and 3.3 per cent after treatment on the 8th and 9th day, respectively; encephalocele in 3.9, 15.5, and 3.3 per cent after treatment on the 7th, 8th, and 9th day, respectively; and hydrocephaly in 49.0, 75.0, 47.5, and 78.7 per cent after treatment on the 7th, 8th, 9th, and 10th day, respectively.

The author reported that rabbit anti-rat-placental antiserum was also teratogenic, and the incidence and severity of the embryonic malformations resembled those produced by kidney antiserum given on the same day of pregnancy.

Mercier-Parot and her collaborators (1963) conducted a similar investigation on pregnant mice. They gave a single intravenous injection of rabbit anti-mouse-kidney antiserum to female Swiss Albino mice on the 6th to 11th day of pregnancy.

The teratogenic period was shorter in the mice than in the rats, with embryonic malformations resulting only after treatment on the 8th and 9th day of pregnancy. Exencephaly was the only CNS malformation reported, and varying degrees of this anomaly were produced solely by treatment on the 8th day of pregnancy.

In this same study, an intravenous injection of rabbit anti-rat-kidney antiserum was also given to female mice on the 8th to 11th day of pregnancy, but it induced no embryonic malformations. This suggested that some form of specific species response might exist.

Slotnick and Brent (1966) used fluorescent antibody techniques to determine the localization of teratogenic anti-rat-kidney and placental antisera in maternal and embryonic rat tissues. They found that these antisera became localized in the basement membrane of the vascular endothelium of several maternal tissues, including the kidneys (glomeruli), adrenals (cortex), liver (central and hepatic veins), and spleen (germinal

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centers of the white pulp). However, the only embryonic site where localization occurred was in the basement membrane of the parietal layer of the yolk sac.

These data and other evidence that the teratogenic factors of antisera are associated with gamma globulins (Brent, 1966), lend support to the idea that the mechanism of action of teratogenic antisera may be related to an immunological disturbance of the function of the yolk sac.

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APPROACH TO THE PROBLEMS

In the present work, myelin basic proteins from the CNS of Long-Evans rats were studied in regard to their development with age and their ability to produce EAE, protect against EAE, and induce teratogenic effects.

The investigations were divided into two general areas - biochemical and histological.

The biochemical studies consisted of two types of experiments. The first, dealt with the electrophoretic assays of basic proteins extracted from myelin and whole brains of immature and mature rats. The second, was concerned with the determinations of total protein content in brain myelin from maturing rats.

The histological studies covered three fields of investigation - the induction of EAE, the production of encephalitogenic tolerance, and the induction of fetal abnormalities.

In the EAE investigations, mature rats were sensitized with complete Freund's adjuvant containing various amounts of two basic proteins from rat brain myelin, one basic protein from bovine spinal cord myelin, one basic protein from guinea pig spinal cord myelin, or whole wet guinea pig spinal cord.

In the encephalitogenic tolerance studies, a series of experiments were conducted to investigate whether rats, which were the offspring of mothers sensitized with two rat myelin basic proteins on the 4th day (preimplantation) or 8th day (postimplantation) of pregnancy, would show a decreased ability to develop EAE lesions when tested at an adult age. These studies were undertaken in view of two earlier reports (Paterson,

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1958; Waksman, 1959b) which indicated that sensitization of newborn rats with whole spinal cord results in a decreased capacity of these animals to develop EAE lesions when tested at mature ages. The findings from these investigations led to the hypothesis that the adult animal's ability to develop EAE depends on its failure to acquire tolerance to the myelin antigen, the antigen being in such small amounts during the early period of development that it fails to affect the immune apparatus.

In the fetal studies, experiments were carried out to determine if maternal sensitization with two rat myelin basic proteins on the 4th day (preimplantation) or 8th day (postimplantation) of pregnancy would produce teratogenic effects in the developing nervous system of young rats. These studies were undertaken in light of three previous investigations (Gluecksohn-Waelsch, 1957; Barber et al., 1959, 1961) which showed that different CNS defects may be induced in mouse embryos as a result of maternal sensitization with whole brain tissue. The researchers suggested that the abnormalities observed in their experiments ultimately might be the result of specific or non-specific antibody responses.

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MATERIALS AND METHODS

I. Biochemical Studies

A. Animals and Brain Samples.

Virgin female Long-Evans rats of various postnatal ages (days 1, 5, 7, 9, 10, 12, 14, 16, 18, 20, 24, 30, 50, 70, 90, 110, 130) were used as test animals. The rats were weighed, anaesthetized with ether, exsanguinated, and decapitated at an upper cervical level. A dorsal mid-line incision was made and skin, muscle, and fascia were stripped away. The calvaria was opened, the olfactory bulbs removed, and the cranial nerves severed at their point of exit in the skull. The brain, including the medulla oblongata, was rapidly extracted and placed in a glass dish which was kept on ice. Next, the brain was rinsed with cold physiologic saline solution and distilled water, stripped of grossly visible blood vessels, blotted free of moisture, weighed, and frozen on dry ice. Each brain sample was kept in a deep-freeze below $0^{o}C$ until it was used for analysis.

B. Preparation of Myelin.

Myelin from the brains of the test animals was prepared by modifications of the ultracentrifugation technique described by Suzuki, Poduslo, and Norton (1967). All of the procedures for the isolation of the myelin were carried out at $0\text{-}4^{o}C$. A 5-7% homogenate of whole brains in cold 0.32 M sucrose was prepared in a Potter-Elvehjem type tissue grinder with a Teflon pestle. The homogenate was layered with a syringe over cold 0.85 M sucrose in nitrocellulose tubes and centrifuged in a Beckman-Spinco Model E (SW-27 rotor) ultracentrifuge at 90,000 x g for

90 minutes. The myelin layer, which was concentrated at the interface of the two solutions, was removed with a Pasteur pipette to clean nitrocellulose tubes, dispersed in cold distilled water, and centrifuged in the SW-27 rotor at 90,000 x g for 30 minutes. The resulting myelin pellets were dispersed in cold distilled water in clean plastic tubes and centrifuged in a Sorvall ultracentrifuge at 30,000 x g for 15 minutes (repeated 3 times). The myelin pellets were resuspended in cold 0.32 M sucrose, layered over cold 0.85 M sucrose in clean nitrocellulose tubes, and centrifuged in the SW-27 rotor at 90,000 x g for 60 minutes. The myelin layer at the interface was removed to clean plastic tubes with a Pasteur pipette, dispersed in cold distilled water, and centrifuged at 30,000 x g for 12 minutes in the Sorvall ultracentrifuge. This last washing step with distilled water was repeated 5 times in order to remove the sucrose.

The wet weight of the myelin was recorded. The myelin was freeze-dried (lyophilized) and the dry weight recorded. Each freeze-dried sample was stored as dry powder over Drierite below $0^{o}C$. All of the samples of myelin for electron microscopic examination were removed from the washed pellet before lyophilization.

C. Electron Microscopy.

Myelin from the brains of 110, 14, and 9 day old postnatal rats were prepared for electron microscopic examination. Each sample was fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate for 1 hour, rinsed in buffer, and postfixed in osmium tetroxide for 1 hour. The sample was dehydrated in a graded series of alcohols and propylene oxide and embedded in Epon 812. The sections were cut on a LKB ultratome with a diamond knife, mounted on naked grids, and stained with uranyl acetate and

Reynold's lead citrate. The micrographs were taken with a Siemens 1. (All of the electron microscopic work was kindly carried out by Ms. Susan Hamamoto Friend, Cancer Research Laboratory, University of California, Berkeley, California.)

D. Extraction of Basic Proteins.

Basic proteins were extracted from freeze-dried myelin and whole wet brains of immature and mature rats. The technique consisted of the removal of lipids with chloroform-methanol and simultaneous extraction of the basic proteins with HCl.

A Polytron was used to homogenize the dried myelin or the whole brains in cold chloroform-methanol (2:1, v/v) and cold pH 1.8 HCl. The homogenate was transferred to a cold glass cylinder and kept at 4°C overnight. The upper phase was removed with a Pasteur pipette, placed in a cold Corex tube, and centrifuged at 4°C in a Beckman-Spinco Model E (SW-27 rotor) ultracentrifuge at 30,000 x g for 30 minutes. The clear supernatant was Pasteur pipetted into dialysis tubing (previously soaked in cold distilled water) and dialyzed against 4 liters of cold 0.1 M HCl for 6 hours (3 changes of HCl) at 4°C. The retentate of basic proteins was freeze-dried (lyophilized) and the dry weight was recorded. Each freeze-dried sample was stored as dry powder over Drierite below 0°C.

E. Electrophoresis.

Polyacrylamide disc electrophoresis was used to assay the freeze-dried basic proteins from myelin and whole brains of immature and mature rats. Electrophoresis for a pH 4.3, 15% gel system was done in the manner described by Reisfeld, Lewis, and Williams (1962), with the following modifications. The electrophoresis was carried out by applying

a current of 6 m. amp. per gel for 90 to 120 minutes. The gels were stained with 0.5% Amido black in 7.5% acetic acid for 90 minutes and destained electrophoretically for 90 or 120 minutes.

Phenol-acetic acid-water, polyacrylamide flat electrophoresis was employed in attempts to demonstrate the basic proteins and proteolipids in dried myelin from immature and mature rat brains. The polyacrylamide gel was cut into square slabs (10 cm x 8 cm) and soaked for 24 hours in the same solvent mixture, phenol-acetic acid-water (2:1:1, w/v/v) at pH 1.5 as used for electrophoresis. The myelin was dissolved in the above mixture in the ratio of 14:3:3 and electrophoresed for 24 hours at $4^{o}C$. After completion of the electrophoresis, the gels were stained with 5% Amido black dissolved in acetic acid, methanol, and water (1:4:5, v/v/v).

Several unsuccessful attempts were made to perfect a phenol-acetic acid-water, polyacrylamide disc electrophoresis technique for the demonstration of the basic proteins and proteolipids in dried myelin from immature and mature rat brains. The procedures which were carried out were based on modifications of a technique described by Cotman and Mahler (1967).

F. Determination of Total Protein Content in Myelin.

The total protein content in the freeze-dried myelin from immature and mature rat brains was determined by modifications of a method described by Lowry, Rosebrough, Farr, and Randall (1951). Each myelin sample was incubated in 1 N NaOH for 18 hours at $37^{o}C$. A solution containing 2% Na_2CO_3 in 0.10 N NaOH, 1% $CuSO_4$, and 2% Na, K tartrate was added to the sample. It was mixed well and allowed to stand for 10 minutes at room temperature. Dilute Folin-Ciocalteu phenol reagent was

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added rapidly and mixed instantaneously. After 30 minutes, the sample was read in a Bausch & Lomb Spectronic 20 Colorimeter/Spectrophotometer at 660 mμ. All of the determinations were carried out in triplicate with Armour Standard bovine serum albumin as the reference standard.

G. Quantitative Determination of Basic Proteins by Densitometry.

Densitometric studies on the electrophoretic patterns of the myelin basic proteins from rat brains were commenced, but they were ultimately discontinued because of the lack of adequate testing equipment.

II. Histological Studies

A. General Animal Care.

Adult virgin female and male rats of the Long-Evans strain were used for all of the experiments except the fetal studies, for which Long-Evans fetal rats aged 21 days were employed.

The adult experimental animals were kept singly or in pairs in stainless steel suspended cages and were fed a standard diet of food pellets (Simonsen White Diet I) and tap water ad libitum; this diet was supplemented with lettuce two times weekly.

B. Antigen-adjuvant Inocula and Injection of Animals.

The antigens used for sensitization were two basic proteins from rat brain myelin, one basic protein from bovine spinal cord myelin, one basic protein from guinea pig spinal cord myelin, and whole wet guinea pig spinal cord.

The basic proteins used as antigens were extracted from adult animal myelin which was prepared by ultracentrifugation. The extraction technique consisted of the removal of lipids with chloroform-methanol

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(2:1, v/v) and simultaneous extraction of the basic proteins with HCl at pH 1.8. The bovine and guinea pig basic proteins were purified by chromatography on Sephadex G-75 and CM-cellulose columns. Polyacrylamide disc electrophoresis (15% gel in β-alanine and acetic acid buffer) was employed to assay the freeze-dried basic proteins. (The preparations and purifications of the bovine and guinea pig basic proteins were kindly carried out by the late Mr. William J. Davis, Department of Neurology, University of California School of Medicine, San Francisco, California.)

The inocula for the non-pregnant rats consisted of the antigen dissolved in one part physiologic saline solution, one part emulsifying agent (Aquaphor)*, and two parts mineral oil+ containing 4 mg/ml of Mycobacterium butyricum‡ (complete Freund's adjuvant). The inocula for the pregnant rats consisted of the antigen dissolved in one part physiologic saline solution, one part emulsifying agent (Aquaphor), and two parts mineral oil (incomplete Freund's adjuvant omitting mycobacteria). The antigen was administered to the pregnant rats in incomplete Freund's adjuvant in order to prevent paralysis and death from occurring in these animals. The control rats received inocula containing physiologic saline solution in complete or incomplete Freund's adjuvant. The emulsions were heated at 60°C, homogenized with a Polytron, and injected into the cutaneous pads on the soles of the hind feet. All of the injections were administered in the morning with a 25 gauge needle attached to a 1 cc tuberculin syringe.

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* Duke Laboratories, Inc., South Norwalk, Connecticut.
\+ Standard Oil Company of California, San Francisco, California.
‡ Difco Laboratories, Detroit, Michigan.

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C. Experimental Allergic Encephalomyelitis (EAE) Studies.

1. Injection of animals.

Female and male rats, 130 days of age, were used as test animals. The weight-ranges of the female and male rats were 240-310 grams and 312-476 grams, respectively. The animals were separated into the following groups:

Rat basic proteins inocula

a. Twelve female rats were inoculated in the right foot pad. Each animal received one injection of 0.1 ml complete Freund's adjuvant containing 43.2 µg of basic proteins.

b. Six male rats were inoculated in the right foot pad. Each animal received one injection of 0.2 ml complete Freund's adjuvant containing 131.2 µg of basic proteins.

c. Six male rats were inoculated with a total amount of 262.4 µg of basic proteins. Each animal received one injection of 0.2 ml complete Freund's adjuvant containing 131.2 µg of basic proteins in the right and left foot pad.

Bovine basic protein inocula

a. Twelve female rats were inoculated in the right foot pad. Each animal received one injection of 0.1 ml complete Freund's adjuvant containing 18.8 µg of basic protein.

Guinea pig basic protein inocula

a. Six female rats were inoculated in the right foot pad. Each animal received one injection of 0.1 ml complete Freund's adjuvant containing 120 µg of basic protein.

b. Six female rats were inoculated in the right foot pad. Each animal received one injection of 0.2 ml complete Freund's adjuvant containing 240 µg of basic protein.

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Guinea pig spinal cord inocula

a. Six female rats were inoculated in the right foot pad. Each animal received one injection of 0.2 ml complete Freund's adjuvant containing 8,000 µg of whole wet spinal cord (theoretically 38.4 µg of basic protein).

Controls

a. Four rats, 2 females and 2 males, were inoculated in the right foot pad. Each animal received one injection of 0.2 ml incomplete Freund's adjuvant containing physiologic saline solution.

b. Four rats, 2 females and 2 males, were inoculated in the right foot pad. Each animal received one injection of 0.2 ml complete Freund's adjuvant containing physiologic saline solution.

c. Four rats, 2 females and 2 males, received no inoculations.

2. Autopsy procedures.

All animals in the EAE studies were weighed daily and checked for clinical evidence of EAE, such as weight loss, ataxic gait, impaired righting reflexes, tremors, loss of tonus, and paralysis of the hind legs.

The animals were sacrificed at the onset of paralysis, or within 2 days of onset by exsanguination from the neck vessels under light ether anesthesia. Rats without clinical symptoms were sacrificed 22-50 days after inoculation.

3. Histological procedures.

The brains, excluding olfactory bulbs, and spinal cords

were rapidly removed, fixed in neutral buffered 10% formalin, and stored in 70% alcohol. Brain sections from the area of the cerebrum, cerebellum-pons, and medulla and spinal cord sections from the cervico-thoracic area were dehydrated through graded alcohols, cleared in chloroform, and infiltrated and embedded in paraffin (M.P. 56-58^{o}C). The paraffin blocks were sectioned at 10 microns. Four cross sections from each sample block were stained with Harris hematoxylin and eosin and examined microscopically for lesions of EAE. In the experimental samples which showed microscopic evidence of EAE lesions and in the control samples, adjacent cross sections were stained with Luxol fast blue for myelin studies.

D. Encephalitogenic Tolerance Studies.

1. Injection of pregnant animals.

Twelve female rats with an age-range of 154-168 days and a weight-range of 223-308 grams were bred with normal males. The day on which a plug of spermatozoa was found in the vagina was considered to be day 0 of pregnancy.

The pregnant rats were separated into two groups. One group consisted of 6 animals which were inoculated on the 4th day of pregnancy. The other group consisted of 6 animals which were inoculated on the 8th day of pregnancy. Each animal in both groups received one injection of 0.1 ml incomplete Freund's adjuvant containing 43.2 µg of rat basic proteins in the right foot pad.

2. Injection of offspring.

Forty-two female and 20 male offspring from both groups

of inoculated pregnant animals were allowed to mature. During the maturation period, the animals were weighed at regular intervals and observed for neurological disorders.

Female and male offspring with an age-range of 130-134 days and weight-ranges of 230-301 grams and 348-468 grams, respectively, were used as test animals. The animals were separated into the following groups:

Rat basic proteins inocula

a. Ten female rats (5 representatives from each group of pregnant animals) were inoculated in the right foot pad. Each animal received one injection of 0.1 ml complete Freund's adjuvant containing 43.2 µg of basic proteins.
b. Ten female rats (5 representatives from each group of pregnant animals) were inoculated in the right foot pad. Each animal received one injection of 0.2 ml complete Freund's adjuvant containing 86.4 µg of basic proteins.
c. Ten female rats (5 representatives from each group of pregnant animals) were inoculated in the right foot pad. Each animal received one injection of 0.1 ml complete Freund's adjuvant containing 129.6 µg of basic proteins.
d. Ten female rats (5 representatives from each group of pregnant animals) were inoculated in the right foot pad. Each animal received one injection of 0.2 ml complete Freund's adjuvant containing 259.2 µg of basic proteins.

Guinea pig spinal cord inocula

a. Six male rats (3 representatives from each group of pregnant animals) were inoculated in the right foot pad. Each animal received one injection of 0.2 ml complete Freund's

adjuvant containing 8,000 µg of whole wet spinal cord (theoretically 38.4 µg of basic protein).

b. Six male rats (3 representatives from each group of pregnand animals) were inoculated in the right foot pad. Each animal received one injection of 0.25 ml complete Freund's adjuvant containing 10,000 µg of whole wet spinal cord (theoretically 48.0 µg of basic protein).

Controls

a. Ten rats (1 female and 4 male representatives from each group of pregnant animals) received no inoculations.

3. Autopsy procedures.

All animals were weighed daily and the inoculated animals were checked for clinical evidence of EAE (See page 53).

Mothers of the offspring were sacrificed after weaning of the young. Mature offspring without clinical symptoms of EAE were sacrificed 22-26 days after inoculation.

4. Histological procedures.

The brains of the offspring were blotted free of moisture and weighed prior to fixation. The brains, excluding olfactory bulbs, and spinal cords from the mothers and offspring were fixed and prepared for microscopic examination in accord with the procedure described in the EAE Studies (Section C).

E. Fetal Studies.

1. Injection of pregnant animals.

Twenty-two female rats with an age-range of 128-147 days and a weight-range of 250-341 grams were bred with normal males.

The day on which a plug of spermatozoa was found in the vagina was considered to be day 0 of pregnancy.

The pregnant rats were separated into two experimental groups and three control groups. The experimental groups consisted of 6 animals which were inoculated on the 4th day of pregnancy and 6 animals which were inoculated on the 8th day of pregnancy. Each animal received one injection of 0.2 ml incomplete Freund's adjuvant containing 131.2 µg of rat basic proteins in the right foot pad.

The control groups consisted of 4 animals which were inoculated on the 4th day of pregnancy and 4 animals which were inoculated on the 8th day of pregnancy. Each animal received one injection of 0.2 ml incomplete Freund's adjuvant containing physiologic saline solution in the right foot pad. Two control animals received no inoculations.

2. Autopsy procedures.

All animals were weighed daily and the inoculated animals were checked for clinical evidence of EAE (See page 53).

All animals were sacrificed on the 21st day of pregnancy, one day before parturition. Each animal was placed under light anesthesia and the abdominal cavity was opened. The implantation sites were counted and the living fetuses were removed, counted, weighed, and inspected for gross abnormalities. The fetuses were placed in Bouin's solution for fixation.

Each animal was sacrificed by exsanguination from the neck vessels. The brain, excluding olfactory bulbs, and spinal cord were rapidly removed and placed in neutral buffered 10% formalin

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for fixation.

3. Examination and dissection of fetuses.

After fixation in Bouin's solution, the fetuses were rinsed and stored in 70% alcohol. Each fetus was measured (crown-rump length and trans-umbilical diameter) and inspected for gross abnormalities of the eyes, face, head, trunk, tail, and limbs. The corners of the mouth were cut to facilitate the examination of the palate and tongue. Two coronal cuts were made through the head to inspect the eyes and brain in those specimens which were not to be examined microscopically. The abdominal and pelvic cavities were opened and examined, and one kidney was cut in cross section. Lastly, the thorax was opened and the thymus and heart inspected. In total, 109 control and 125 experimental fetuses were examined in this manner.

4. Histological procedures.

The brains and spinal cords from the pregnant animals were prepared for microscopic examination in accord with the procedures described in the EAE Studies (Section C).

In view of the fact that none of the fetuses showed abnormalities, the head of only one fetus from each pregnant animal was prepared for microscopic examination. Each head was decalcified in 5% nitric acid in 80% alcohol, skinned, and cut in half frontally. The cut halves of each head were dehydrated through graded alcohols, cleared in terpineol, and infiltrated and embedded in paraffin (M.P. 56-58^{o}C). The entire infiltration period was carried out under vacuum. The

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paraffin blocks were serially sectioned at 8 microns, and each 30th section was stained with Harris hematoxylin and eosin.

RESULTS

I. Biochemical Studies

A. Electron Microscopic Assessment of Myelin Purity.

The electron microscopic examinations of myelin preparations from the brains of 110, 14, and 9 day old postnatal Long-Evans rats indicated that these samples were free of subcellular structures other than myelin. The structural pattern of the myelin was identical with that seen in normal CNS white matter. It was arranged in rings or lamellae containing a varying number of sheaths and the number of sheaths increased progressively with the maturity of the myelin samples. Each sheath was characterized by two dense lines, forming the main periods, and a lighter staining intraperiod line. Splitting and unwinding occurred at the intraperiod lines. In some areas, the section was tangential to the plane of the myelin lamellae and as a result, the periodic patterns were unrecognizable. Occasionally, dense granular configurations of inorganic debris were present (Figures 1, 2, and 3).

B. Myelin Preparations and Yields.

The brains of postnatal Long-Evans rats, ranging in age from 1 to 130 days, were used for the preparation of myelin by ultracentrifugation techniques. The wet weights of the individual brains from animals of the same age were quite uniform. In general, there was a progressive increase in the wet brain weights of the maturing animals up to approximately 90 days of age.

The initial ultracentrifugation studies showed that it was very difficult to isolate myelin from the brains of 1, 5, 7, and 9 day old

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rats. Thereafter, the brains from 10 day old rats were used as the earliest age for the preparation of myelin.

Approximately 26 grams of whole brain tissue (wet weight) were used for the myelin preparations for each age group since it was found that an average of 12 grams of whole brain tissue (wet weight) did not result in a sufficient yield of myelin for the earlier age groups. In general, there was a progressive increase in the yields of myelin (dry weight) for each age group (Table I). The myelin yields were extremely low in the very early age groups.

The average yield of basic proteins extracted at pH 1.8 from adult myelin preparations was approximately 1 mg proteins per 20 mg myelin (dry weight). Not much change could be detected in the yields of basic proteins from animals between 20 and 130 days of age, however, smaller yields were obtained from animals younger than 20 days of age. Wood (personal communication) reported a similar average yield for the myelin basic proteins isolated from adult rats. He also did not observe significant differences in the values of the basic proteins from animals between 20 and 160 days of age.

C. Electrophoretic Assays of Myelin Basic Proteins.

The polyacrylamide disc electrophoresis studies (15% gels at pH 4.3) on basic protein extracts (pH 1.8) from myelin preparations from single or pooled numbers of brains of Long-Evans rats at 130 days of age showed that two major basic proteins were present in the CNS myelin of this strain of rats (Figure 4). The larger basic protein, which had a slower electrophoretic mobility, was comparable to the single encephalitogenic basic protein found in guinea pig, rabbit, bovine, monkey, and human CNS myelin. The smaller basic protein, which had a faster cathodic mobility,

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TABLE I.

Myelin Yields from Developing Rat Brains

Age of Rats (postnatal)	Total Number of Brains	Total Wet Weight of Brains	Total Dry Weight of Myelin	Average Dry Weight of Myelin per Brain
10 days	28	26.37 g	57.60 mg	2.06 mg
12 days	24	26.26 g	71.25 mg	2.97 mg
14 days	22	26.32 g	102.80 mg	4.67 mg
16 days	20	26.34 g	149.65 mg	7.48 mg
18 days	19.5	26.31 g	221.25 mg	11.35 mg
20 days	19	26.12 g	302.80 mg	15.94 mg
24 days	18	25.88 g	361.60 mg	20.09 mg
30 days	17	25.97 g	639.45 mg	37.61 mg
50 days	15	25.92 g	889.55 mg	59.30 mg
70 days	14.5	25.95 g	934.30 mg	64.43 mg
90 days	14	25.87 g	1,039.20 mg	74.23 mg
110 days	14	25.93 g	1,096.35 mg	78.31 mg
130 days	14	25.91 g	1,147.30 mg	81.95 mg

was characteristic for the rat. The different staining intensities of the two electrophoretic protein bands indicated that there was proportionally more of the smaller basic protein than the larger basic protein.

The polyacrylamide disc electrophoresis technique (15% gels at pH 4.3) was used to compare the basic protein extracts (pH 1.8) from myelin preparations from single brains of rats at 18, 24, and 130 days of age, respectively. The equipment was not sensitive enought to detect the very small quantities of the two basic proteins in the 18 day old preparations; however, each of the two basic proteins were demonstrable as a distinct electrophoretic band in the 24 day old preparations. The staining intensities of the two protein bands indicated that there were greater amounts of both basic proteins in the 130 day old preparations than in the 24 day old preparations and during this period of maturation there was a larger proportional increase in the smaller basic protein than in the larger basic protein (Figure 5).

More extensive polyacrylamide disc electrophoresis studies (15% gels at pH 4.3) were conducted on basic protein extracts (pH 1.8) of myelin prepared from a pooled number of brains from rats at 14, 16, 18, 20, 24, 30, 50, 70, 90, 110, and 130 days of age. These studies showed that both the larger and smaller basic proteins were present as single, distinct electrophoretic bands as early as 14 days after birth (Figure 6). The staining intensities of the two protein bands again indicated that during brain maturation there was an increase in both of the basic proteins, and proportionally there was a greater increase in the smaller basic protein than in the larger basic protein.

The same type of polyacrylamide disc electrophoresis studies (15% gels at pH 4.3) which were carried out using basic proteins extracted at pH 1.8 from single whole brains of rats at 1, 5, 7, 9, 12, 14, 18, 24,

and 130 days of age suggested that the two myelin basic proteins were probably present earlier than 14 days after birth (Figure 7). Although the close proximity of some of the protein bands in this particular study led to some difficulties in identifying certain proteins in the younger brain samples, a faint electrophoretic band which appeared to correspond with the larger myelin basic protein was detectable at 1 day after birth. Two faint electrophoretic bands, which were very close to each other, were observed in the region of the smaller myelin basic protein in the 1 and 5 day old brain samples. At 7 days after birth, these two protein bands appeared to merge into a single band which corresponded with the smaller myelin basic protein found in the mature rat brain. The different electrophoretic staining intensities of the two myelin basic proteins indicated that proportionally there was a greater increase in the smaller basic protein than in the larger basic protein during brain maturation. In addition, the increase in the two myelin basic proteins appeared to coincide with the reduction of some other basic proteins in the maturing brain.

The phenol-acetic acid-water, polyacrylamide flat electrophoresis technique which was employed in attempts to demonstrate the basic proteins and the proteolipid protein in myelin from immature and mature rat brains did not provide satisfactory results because the smaller myelin basic protein could not be detected. However, when myelin brain samples from rats at 18, 24, and 130 days of age were tested by this technique, there was electrophoretic staining evidence of an increase in the larger myelin basic protein and the proteolipid protein during brain maturation.

Several attempts were made to perfect a phenol-acetic acid-water, polyacrylamide disc electrophoresis technique for demonstrating the basic

proteins and the proteolipid protein in myelin from immature and mature rat brains. This technique did not result, however, in the production of satisfactory gels with clearly discernible electrophoretic protein bands.

D. Protein Content in Myelin During CNS Development.

The determinations for the total protein content in myelin brain samples from Long-Evans rats at 10, 12, 14, 16, 18, 20, 24, 30, 50, 70, 90, 110, and 130 days of age have been summarized in Table II. The total protein content, which was expressed as the percentage of the dried myelin weight, showed an increase from 7.87 per cent at 10 days to 23.17 per cent at 130 days. Myelin protein was found to accumulate throughout development, but the period of greatest accumulation appeared to occur from 10 to 30 days after birth.

II. Histological Studies

A. Experimental Allergic Encephalomyelitis (EAE) Studies.

The experimental efforts to induce EAE in Long-Evans rats sensitized with complete Freund's adjuvant containing two basic proteins from rat brain myelin, one basic protein from bovine spinal cord myelin, one basic protein from guinea pig spinal cord myelin, or whole wet guinea pig spinal cord have been summarized in Table III. Flaccid paralysis of the hind legs was observed in only 4 of the 54 experimental rats. Two of the 4 animals became paralyzed on the 12th and 13th day after a single injection of 120 µg of guinea pig basic protein in 0.1 ml complete Freund's adjuvant. Paralysis occurred in the other 2 animals on the 12th and 16th day after a single injection of 8,000 µg of whole wet guinea spinal cord in 0.2 ml complete Freund's adjuvant. A weight loss of 17 to 24 grams

TABLE II.

Protein Content in Brain Myelin of Developing Rats

Age of Rats (postnatal)	Total Protein (per cent dry weight)
10 days	7.87
12 days	9.17
14 days	12.01
16 days	13.84
18 days	15.00
20 days	17.50
24 days	18.17
30 days	19.83
50 days	20.33
70 days	21.17
90 days	21.93
110 days	22.67
130 days	23.17

Values are the mean of triplicate determinations differing by no more than 1 per cent.

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TABLE III.

Effects of EAE on Rats After Sensitization with Neuroantigens

Rat Groups	Composition of Sensitizing Inoculum		Total Number of Rats	Number of Rats Affected	
	CNS Antigen	Complete Freund's Adjuvant		Paralysis	CNS Lesions
A.	43.2 µg RBP	0.10 ml	12	0	2
B.	131.2 µg RBP	0.20 ml	6	0	1
C.	262.4 µg RBP	0.40 ml	6	0	1
D.	18.8 µg BBP	0.10 ml	12	0	0
E.	120.0 µg GBP	0.10 ml	6	2	3
F.	240.0 µg GBP	0.20 ml	6	0	1
G.	8,000.0 µg GWC	0.20 ml	6	2	5

KEY: RBP = Rat Basic Proteins.
BBP = Bovine Basic Protein.
GBP = Guinea Pig Basic Protein.
GWC = Guinea Pig Whole Spinal Cord.

preceded the appearance of paralysis in these animals.

All of the experimental animals had highly active inoculation sites in the foot pads which showed progressive enlargement, inflammation, ulceration, and crusting, followed by partial or complete healing.

EAE lesions were demonstrable in the CNS of clinically asymptomatic rats as well as in those with paralysis. CNS lesions were present in at least one rat in each experimental group which was sensitized with complete Freund's adjuvant containing various amounts of rat basic proteins, guinea pig basic protein, or whole guinea pig spinal cord. There was no evidence, however, of any EAE lesions in the group of experimental animals which were sensitized with bovine basic protein in complete Freund's adjuvant.

Guinea pig neuroantigens appeared to be the most effective agents for inducing EAE in this strain of rats since they produced EAE lesions in the greatest number of animals and they were the only neuroantigens which caused clinical evidence of EAE (hind leg paralysis).

The EAE lesions consisted of vascular and perivascular accumulations of mononuclear inflammatory cells. These cells, which were pleomorphic resembled large and small lymphocytes and monocytes, with lymphocytes being the more prevalent cell type. They often aggregated along the endothelium of a blood vessel or filled the lumen of the vessel. In turn, they traversed the vascular wall, accumulated in a perivascular cuff, and spread into the surrounding CNS parenchyma (Figures 8, 9, 10, 11, and 12).

There was variability in the location, number, and size of the CNS lesions in the rats affected with EAE. Lesions were present at various levels of the neuroaxis, but there was a predilection for larger and more numerous lesions in the spinal cord and brain stem (medulla and lower

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pons). All of the 13 EAE affected animals had lesions in these two general areas, whereas 7 had lesions in the cerebellum, and only 4 had lesions in the cerebrum (Table IV). The lesions in the latter two regions tended to be small and few in number.

There was a tendency for EAE lesions to occur in both the white and gray matter of the CNS in the small number of animals with hind leg paralysis, but in the majority of animals, the lesions were distributed in the white matter. Vascular and perivascular infiltrations of mononuclear inflammatory cells were especially apt to be found in the long tracts of the spinal cord white matter and near the area of the fourth ventricle. Foci of inflammatory cells were also frequently present in and beneath the meninges surrounding the spinal cord.

Luxol fast blue stains of sections from the brains and spinal cords of the animals with EAE lesions did not reveal any appreciable injury to myelin.

The control experiments consisted of 4 rats which received a single injection of 0.2 ml complete Freund's adjuvant, 4 rats which received a single injection of 0.2 ml incomplete Freund's adjuvant, and 4 rats which did not receive any inoculations. The inoculation site (foot pad) in the injected animals showed greater enlargement, inflammation, and ulceration after sensitization with complete Freund's adjuvant than after sensitization with incomplete Freund's adjuvant. There was no clinical or histological evidence of EAE in any of the 12 control animals.

B. Encephalitogenic Tolerance Studies.

The present series of experiments were undertaken to investigate whether adult Long-Evans rats, which were offspring of mothers injected

TABLE IV.

Locations of EAE Lesions

Rat Groups (see Table III)		Spinal Cord	Medulla and Pons	Cerebellum	Cerebrum
A.	1.	+	+	-	-
	2.	+	+	+	-
B.	1.	+	+	+	-
C.	1.	+	+	-	-
E.	1.	+	+	-	+
	2.	+	+	-	+
	3.	+	+	+	-
F.	1.	+	+	+	-
G.	1.	+	+	-	-
	2.	+	+	+	+
	3.	+	+	+	-
	4.	+	+	-	-
	5.	+	+	+	+

KEY: + = One or more lesions observed.
- = No lesions observed.

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during pregnancy with two basic proteins from rat brain myelin, would show a decreased capacity to develop EAE lesions after sensitization with rat or guinea pig neuroantigens.

The two experimental groups of 6 female rats which were inoculated with 43.2 µg of rat basic proteins in 0.1 ml incomplete Freund's adjuvant on the 4th and 8th day of pregnancy, respectively, showed no clinical or histological evidence of EAE. All of the animals had moderately active inoculation sites in the foot pads.

A total of 42 female and 20 male offspring from both groups of inoculated pregnant animals were allowed to mature. At 130-134 days of age, the female and male offspring were separated into two experimental groups.

Forty female offspring were divided into four groups of 10 animals (5 representatives from each group of pregnant animals). Each female in the four groups received a single injection of complete Freund's adjuvant containing 43.2 µg, 86.4 µg, 129.6 µg, or 259.2 µg of two basic proteins from rat brain myelin.

Twelve male offspring were divided into two groups of 6 animals (3 representatives from each group of pregnant animals). Each male in the two groups received a single injection of complete Freund's adjuvant containing 8,000 µg or 10,000 µg of whole wet guinea pig spinal cord.

The 52 adult experimental offspring, each of which had highly active inoculation sites in the foot pads, were sacrificed 22-26 days after sensitization. At this time, flaccid paralysis of the hind legs was not observed in any of the offspring; however, EAE lesions were present in at least one offspring in each experimental group sensitized with rat or guinea pig neuroantigens (Tables V and VI). A slightly larger ratio of the offspring had EAE lesions after sensitization with whole guinea pig

TABLE V.

Effects of EAE Induced by Neuroantigens in Adult Offspring from Females Sensitized with RBP on 4th Day of Pregnancy

Rat Groups	Composition of Sensitizing Inoculum		Total Number of Rats	Number of Rats Affected	
	CNS Antigen	Complete Freund's Adjuvant		Paralysis	CNS Lesions
A.	43.2 µg RBP	0.10 ml	5	0	2
B.	86.4 µg RBP	0.20 ml	5	0	1
C.	129.6 µg RBP	0.10 ml	5	0	1
D.	259.2 µg RBP	0.20 ml	5	0	1
E.	8,000.0 µg GWC	0.20 ml	3	0	2
F.	10,000.0 µg GWC	0.25 ml	3	0	2

KEY: RBP = Rat Basic Proteins.
GWC = Guinea Pig Whole Spinal Cord.

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TABLE VI.

Effects of EAE Induced by Neuroantigens in Adult Offspring from Females Sensitized with RBP on 8th Day of Pregnancy

Rat Groups	Composition of Sensitizing Inoculum		Total Number of Rats	Number of Rats Affected	
	CNS Antigen	Complete Freund's Adjuvant		Paralysis	CNS Lesions
A.	43.2 µg RBP	0.10 ml	5	0	1
B.	86.4 µg RBP	0.20 ml	5	0	1
C.	129.6 µg RBP	0.10 ml	5	0	1
D.	259.2 µg RBP	0.20 ml	5	0	1
E.	8,000.0 µg GWC	0.20 ml	3	0	1
F.	10,000.0 µg GWC	0.25 ml	3	0	1

KEY: RBP = Rat Basic Proteins.
GWC = Guinea Pig Whole Spinal Cord.

spinal cord than after sensitization with rat myelin basic proteins.

There were no appreciable differences observed in the offspring from either of the two groups of sensitized pregnant animals in regard to the number of offspring with EAE lesions or the severity of the lesions. The EAE lesions in the affected offspring showed the same basic characteristics (Figure 13, 14, 15, and 16) and the same patterns of distribution (Tables VII and VIII) as the lesions described previously in the EAE Studies (Section A). In addition, Luxol fast blue stains of sections from the brains and spinal cords of the EAE affected offspring did not reveal any appreciable injury to myelin.

The 10 control offspring (1 female and 4 male representatives from each group of pregnant animals) which were allowed to mature did not receive any inoculations. The wet weights of the brains of these offspring were within the normal range and there was no histological evidence of abnormalities in either their brains or spinal cords.

C. Fetal Studies.

The present investigations were undertaken to determine if maternal sensitization with two basic proteins from rat brain myelin would produce teratogenic effects in the developing nervous system of fetal rats.

There was no clinical or histological evidence of EAE in the two experimental groups of 6 female rats which were inoculated with 131.2 µg of rat basic proteins in 0.2 ml incomplete Freund's adjuvant on the 4th and 8th day of pregnancy, respectively. The two control groups of 4 female rats which were inoculated with 0.2 ml incomplete Freund's adjuvant on the 4th and 8th day of pregnancy, respectively, also showed no clinical or histological evidence of EAE. All of the inoculated animals had moderately active inoculation sites in the foot pads.

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TABLE VII.

Locations of EAE Lesions in Adult Offspring

Rat Group (see Table V)		Spinal Cord	Medulla and Pons	Cerebellum	Cerebrum
A.	1.	+	+	+	-
	2.	+	+	-	+
B.	1.	+	+	+	-
C.	1.	+	-	-	-
D.	1.	+	+	-	+
E.	1.	+	+	+	-
	2.	+	+	-	-
F.	1.	+	+	-	-
	2.	+	+	+	+

KEY: + = One or more lesions observed.
- = No lesions observed.

TABLE VIII.

Locations of EAE Lesions in Adult Offspring

Rat Groups (see Table VI)		Spinal Cord	Medulla and Pons	Cerebellum	Cerebrum
A.	1.	+	+	-	-
B.	1.	+	+	+	+
C.	1.	+	+	+	-
D.	1.	+	+	-	-
E.	1.	+	+	+	+
F.	1.	+	+	-	+

KEY: + = One or more lesions observed.
- = No lesions observed.

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The experimental and control animals were sacrificed on the 21st day of pregnancy, one day before parturition. The number and weights of the fetuses for each mother were within the normal ranges.

One hundred and twenty-five experimental and 109 control fetuses were inspected for gross and internal abnormalites. All of the fetuses had crown-rump lengths and trans-umbilical diameters within the normal ranges. They showed no evident gross abnormalities of the eyes, face, head, trunk, tail, and limbs. In addition, they exhibited no apparent internal abnormalities in the areas of the head, thorax, abdomen, and pelvis.

In view of the fact that none of the fetuses showed abnormalities, the head of only one fetus from each pregnant animal was prepared for microscopic examination. There was no histological evidence of abnormalities in the development of the skull, brain, eyes, ears, mouth, and nose in the fetal heads (12 experimentals and 3 controls) which were serially sectioned and stained with hematoxylin and eosin.

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DISCUSSION

I. Biochemical Studies

A. Electrophoretic Assays of Basic Proteins in Adult Myelin.

In the present studies, electrophoretic analyses of protein extracts (pH 1.8) of CNS myelin from Long-Evans rats in 15% polyacrylamide gels (pH 4.3) have shown that two major basic protein components exist in the CNS myelin of this strain of rats.

Several workers have reported the presence of two basic proteins in the CNS myelin of other strains of rats (Eng et al., 1968; Martenson et al., 1969, 1970a, 1970b, 1971a, 1971b; Martenson and Gaitonde, 1969a, 1969b; Agrawal et al., 1970; Gaitonde and Martenson, 1970; Kies, 1970; Mehl and Halaris, 1970; Sammeck et al., 1971; Wood and King, 1971; Quarles et al., 1973; Druse et al., 1974; Dunkley and Carnegie, 1974) as well as of mice (Greenfield et al., 1971; Martenson et al., 1971a; Morell et al., 1972; Matthieu et al., 1973), hamsters, squirrels, prairie dogs, and woodchucks (Martenson et al., 1971a).

In all of these rodents, the larger basic protein, which has a slower electrophoretic mobility, is comparable to the single encephalitogenic myelin basic protein which exists in other mammalian species. The smaller encephalitogenic basic protein, which is unique for the foregoing rodents, has a faster cathodic mobility.

Quantitative densitometric measurements were not included in the present work, but the different staining intensities of the two electrophoretic protein bands suggest that there is proportionally less of the larger basic protein than the smaller basic protein in adult rat myelin.

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Studies by other researchers have provided definite quantitative evidence that in adult rats there is less of the larger basic protein than the smaller basic protein. The ratio of the two proteins varied between 1:1.75 and 1:3 according to whether the proteins were extracted from isolated myelin (Sammeck et al., 1971; Adams and Osborne, 1973) or whole brain tissue (Martenson et al., 1970a; Eng et al., 1971). Sammeck and his co-workers (1971) reported the ratio did not vary in samples from different areas of the CNS in adult rats. Two recent studies have indicated that during the ontogenetic development of CNS myelin in rats, there is a markedly proportional decrease in the larger basic protein and a large relative increase in the smaller basic protein (Adams and Osborne, 1973; Zgorzalewicz et al., 1974).

Biochemical investigations have shown that the larger encephalitogenic rat basic protein is comparable in size (approximately 18,400 daltons) with the single encephalitogenic basic protein found in the majority of other species (Martenson et al., 1972a, 1972b). The additional encephalitogenic basic protein in rats is smaller by approximately 4,000 daltons (Martenson et al., 1972b; Kies et al., 1972).

Preliminary studies by researchers indicated that the smaller rat basic protein differed from the larger basic protein in having a sequence of 40 amino acid residues missing within the C-terminal half of the molecule (Dunkley, et al., 1972; Martenson et al., 1972b). In 1973, part of the amino acid sequence of an encephalitogenic peptide of the smaller rat basic protein was reported in connection with work on the localization of the antigenic determinants which induce EAE in rats (Dunkley et al., 1973; McFarlin et al., 1973).

Recently, Dunkley and Carnegie (1974) determined the complete amino

acid sequence of the smaller encephalitogenic basic protein from rat brain myelin. They found that the major internal deletion of 40 amino acid residues is equivalent to residues 120-160 in the larger encephalitogenic basic protein which is found in the majority of other species. They stated that this deletion differs from those usually found in other groups of proteins because of its large size and central location in the molecule. They also pointed out that the central position of the deletion shows the smaller basic protein could not be derived from the larger basic protein as a result of proteolysis.

Both of the encephalitogenic basic proteins are present in outbred and inbred strains of rats which favors the hypothesis that they originated as products of non-allelic genes (Martenson et al., 1971a). Dunkley and Carnegie (1974) have suggested that the smaller basic protein gene was undoubtedly produced by a duplication process, and the shorter length of the product must be the result of removal of a portion of the gene during duplication or at some later time.

In addition, these investigators also described two possible mechanisms which could result in the formation of the smaller basic protein gene. The first would involve unequal crossover during synapsis at partially homologous DNA regions. Bauer (1972) stated that several regions of the basic protein, including amino acid residues 114-124 and 154-164, have a homology with an "ancestral histone IV gene". An alignment error of these regions during synapsis could lead to a new gene which would code for a protein equivalent in sequence and size to the smaller basic protein. The second possible mechanism for the formation of the smaller basic protein gene might involve misrepair of the DNA coding for the larger basic protein following breakage by either enzymatic or mechani-

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cal processes.

Dunkley and Carnegie (1974) considered that the sequence of amino acids in the smaller rat basic protein shows a close homology with the encephalitogenic basic proteins of other species, and a large proportion of the basic protein appears to have an invarient sequence.

One conservative region in the basic protein (residues 90-120) contains a number of unusual features: a methylated arginine residue (109), a triproline sequence (101-103), a serine residue (112) that can be phosphorylated in vitro (Carnegie et al., 1973), a threonine residue (100) that can be glycosylated in vitro (Hagopian et al., 1971), and a tryptophan residue (118) that is important for the induction of EAE in guinea pigs and which interacts with serotonin in vitro (Carnegie et al., 1972).

There is little information available on the amino acid sequence of the larger rat basic protein. A preliminary study on tryptic peptides by Martenson and his associates (1972b) indicated that there is a reasonable homology in the amino acid sequence of this protein and the bovine and human encephalitogenic basic proteins. It has been suggested that the 40 amino acid residues which are deleted in the smaller rat basic protein may not be necessary for whatever structural or functional role the protein plays in myelin. Dunkley and Carnegie (1974) pointed out, however, that if this region were unimportant, it would be expected to show variability in its amino acid sequence, and this is not the case. They suggested that perhaps it is advantageous to retain two myelin basic proteins with a common amino acid sequence in order to protect against mutational errors which could lead to a loss of function.

B. Electrophoretic Assays of Myelin Basic Proteins During CNS Development.

In the present series of investigations, the electrophoretic distributions of basic proteins during postnatal brain maturation were studied using myelin and whole brains from Long-Evans rats.

The electrophoretic analysis of protein extracts (pH 1.8) of brain myelin from 14 to 130 day old rats in 15% polyacrylamide gels (pH 4.3) showed that both the larger and smaller encephalitogenic basic proteins are present as single, distinct protein bands as early as 14 days of age in this strain of rats.

The same type of polyacrylamide electrophoresis studies on basic proteins extracted at pH 1.8 from single, whole brains of 1, 5, 7, 9, 12, 14, 18, 24, and 130 day old rats, demonstrated that during brain maturation new basic proteins are formed along with the simultaneous disappearance of other basic proteins. The increase in the two encephalitogenic basic proteins appeared to coincide with the reduction of other basic proteins.

The close proximity of some of the basic protein bands in this particular study led to some difficulties in identifying certain proteins in the younger brain samples. A faint electrophoretic band which appeared to correspond with the larger, slower moving encephalitogenic basic protein was detectable at 1 day after birth. Two faint electrophoretic bands, which were very close to each other, could be observed in the region of the smaller, faster moving encephalitogenic basic protein in the 1 and 5 day old brains. At 7 days, these two protein bands appeared to merge into a single band corresponding with the smaller encephalitogenic basic protein found in the mature rat brain.

The different electrophoretic staining intensities of the two

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encephalitogenic basic proteins in both the myelin and whole brain studies appeared to indicate that the two basic proteins increase in amount during CNS maturation, but proportionally there is a greater increase in the smaller basic protein than in the larger basic protein.

Observations pertaining to the electrophoretic distribution of basic proteins during postnatal CNS maturation have been described in recent years in five mammalian species. Thus, in studies with various strains of rats, the two encephalitogenic myelin basic proteins have been reported to be present as single, distinct electrophoretic bands in the immature brain of this species at 16 (Eng et al., 1968), 15 (Gaitonde and Martenson, 1970), 14 (Agrawal et al., 1972; Druse et al., 1974), and 12 (Adams and Osborne, 1973) days of age. Wood and King (1971) reported that each of the two encephalitogenic basic proteins was split into two electrophoretic bands in their investigation with 16 day old Lewis rats. In view of the findings of other researchers, this observation was probably the result of enzymatic degradation of the two basic proteins.

Gaitonde and Martenson (1970) reported that neither of the two encephalitogenic basic proteins were detectable in the brain of 5 day old M.R.C. T_3 rats. They noted that the smaller basic protein could be observed in the brain at 10 days after birth, but the larger basic protein could not be identified at this time. They indicated that the presence of the latter protein might have been obscured by other electrophoretically slower moving components.

In a later study, Adams and Osborne (1973) reported they were also unable to detect either of the two encephalitogenic basic proteins in the early brain myelin of 5 day old Wistar rats. At 7 days, they observed an electrophoretic band corresponding with the larger basic protein, but it was not until 11-12 days after birth that the smaller basic protein

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also made its appearance. They stated that the changeover from 'early myelin' to adult myelin occurred at 11-12 days of age.

The myelin basic proteins in all of the foregoing studies were isolated from whole rat brains and, therefore, must be considered as the sum of different developmental stages in the brain, which is known to show differences in the onset of myelination in various areas (Jacobson, 1963).

Recently, Zgorzalewicz and his associates (1974) were able to investigate the basic proteins in separate myelin preparations from the forebrain, cerebellum, and spinal cord of Wistar rats as early as 15, 10, and 5 days after birth, respectively. They reported that the two encephalitogenic basic proteins could be demonstrated electrophoretically in each of the three CNS areas at the different stages of development which were examined.

In studies with different strains of mice, the two encephalitogenic myelin basic proteins have been reported to be present as single, distinct electrophoretic bands in the immature brain at 8 (Morell et al., 1972), 10 (Greenfield et al., 1971), and 15 (Matthieu et al., 1973) days of age. The latter group of researchers stated that they were unable to detect the two myelin basic proteins in electrophoretograms of protein extracts from mouse brains at 1, 5, and 10 days of age.

Einstein and her associates (1970) investigated the electrophoretic distribution of basic proteins in the developing brain and spinal cord of rabbits. In their studies, the earliest time at which the encephalitogenic basic protein could be clearly detected in either the brain or spinal cord was on the 9th day after birth.

Hegstrand and Kornguth (1973) reported that the encephalitogenic

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basic protein could be demonstrated electrophoretically in the brains of fetal calves (30-65 cm crown-rump length). In a more recent investigation, Fewster and her associates (1974) were able to confirm this finding by showing that the encephalitogenic basic protein was present in the white matter of brains from 9 month old fetal calves.

In electrophoretic studies dealing with the maturation of myelin proteins in the human brain, two groups of workers reported that they were unable to observe the encephalitogenic basic protein in the brains of fetuses and infants (Einstein and Csejtey, 1966; Savolainen et al., 1972). In contrast with these reports, Eng and his co-workers (1968) noted that an electrophoretic band corresponding with the encephalitogenic basic protein was already present in the brain myelin of an infant at 10 weeks of age. Recently, Fishman and his associates (1975) were able to confirm this finding by demonstrating the presence of the encephalitogenic basic protein in myelin isolated from the brains of newborn infants.

It is evident from the foregoing electrophoretic investigations that although there are species differences with respect to the appearance of the encephalitogenic myelin basic protein(s), in general, the protein(s) is present at early periods in development. In addition, it has also been found there is an increase in the amount of the basic protein(s) during maturation in all of the species which have been examined.

On the whole, the electrophoretic findings concerning the development of the myelin basic protein(s) concur with the data from assay studies pertaining to the encephalitogenic activity of CNS tissue or myelin basic protein from immature animals. In the earliest investigations of this sort, Kabat and his associates (1947, 1948) reported that the spinal cords

of 3 day old rabbits and the brains of 12 day old rabbits were capable of inducing EAE in monkeys. In a later study, Waksman (1959b) showed that the spinal cords of rats at 7 days after birth were able to produce EAE in rats, but the encephalitogenic reaction was weak and did not reach adult levels even at the age of 21 days.

Svet-Moldavskij and his co-workers (1965) used guinea pigs to assay the development of encephalitogenic activity in the CNS of several different species. Their studies showed that the brains of mice were not capable of inducing EAE until the 16th day after birth. At this time the EAE reaction was weak, but it became increasingly stronger as the mice matured. The earliest time at which the brains of rats caused a weak encephalitogenic reaction was the 20th day after birth. This reaction increased progressively in intensity with the increasing maturity of the rats. The rabbit brains were encephalitogenic as early as the 2nd day after birth, but again, this reaction was much weaker than in the more mature animals. In contrast with the foregoing species, the brains of guinea pigs and of sheep were highly encephalitogenic even on the first day after birth. The researchers also reported that the spinal cords in all of the animals tested were already encephalitogenic towards the end of fetal development.

In a 1966 study, Einstein and Csejtey tested the encephalitogenic activity of a basic protein extract from immature human brain in guinea pigs. They found that the protein preparation from a 10 week old infant was not encephalitogenic, but the preparation from an 8 month old infant was capable of inducing EAE in 1 out of 6 of the test animals. Encephalitogenic activity also appeared to increase with age in humans since the protein preparation from a 4 year old child was moderately encephalito-

genic, whereas that from a 6 year old child was highly encephalitogenic.

Some of the foregoing investigations, which have been cited concerning the development of encephalitogenic activity and the appearance of encephalitogenic basic proteins during CNS maturation, contain conflicting data. Much of this is undoubtedly caused by the fact that various investigators have used different criteria for testing encephalitogenic activity, different procedures for isolating myelin and extracting basic proteins, and different techniques for assaying and electrophoresing protein components of the CNS.

In recent years, improved density gradient methods for the isolation of myelin and the advent of new techniques for the solubilization and separation of myelin proteins, notably the sodium dodecyl sulphate electrophoretic methods, have allowed the protein composition of myelin to be described in more detail in adult and developing animals. With respect to the latter animals, several workers (Agrawal et al., 1970, 1973; Morell et al., 1972; Adams and Osborne, 1973; Benjamins et al., 1973) have isolated a myelin-like membrane fraction from the brain tissue of immature rats and mice which differs from purified adult myelin by having large quantities of high molecular weight proteins and small quantities of encephalitogenic basic proteins. Recently, Agrawal and his associates (1974) reported that myelin prepared from immature rat brain tissue can be separated into additional subfractions. Findings such as these have led to the idea that a number of sequentially related phases occur during the process leading to the formation of chemically and structurally mature myelin.

Future studies should be directed towards locating the sites of synthesis of the individual myelin proteins and studying the appearance

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of individual newly formed proteins in myelin and myelin-like fractions from animals at earlier developmental stages than have been studied so far.

C. Protein Content in Myelin During CNS Development.

In the present study, the total protein content in brain myelin of Long-Evans rats at various ages (10 days to 130 days) was estimated by the procedure of Lowry, Rosebrough, Farr, and Randall (1951).

The total protein content, which was expressed as the percentage of the dried myelin weight, showed an increase from 7.87 per cent at 10 days to 23.17 per cent at 130 days. Myelin protein was found to accumulate throughout development, but the period of greatest accumulation appeared to occur from 10 to 30 days after birth. This time period coincides with that of rapid myelination in the CNS of rats and mice (Morell et al., 1972; Norton and Poduslo, 1973). In these two species, there is a continual increase in brain myelin, although at a steadily decreasing rate, for many months after the animals have reached maturity (Morell et al., 1972; Norton and Poduslo, 1973; Smith, 1973).

Other investigators have also reported an increase in the total protein content of brain myelin (per cent dry weight) during development. In studies with rats, Eng and Noble (1968) observed an increase in myelin protein from 10 per cent at 9 days to 22.8 per cent at 90 days, while Petropoulos and his co-workers (1972) noted an increase in myelin protein from 3.66 per cent at 1 day to 21.36 per cent at 70 days.

In similar studies with rabbits, Einstein and her associates (1970) determined the total protein content of spinal cord myelin as well as brain myelin. They reported that in brain myelin there was an increase in protein from 7.03 per cent at 1 day to 22.33 per cent at 170 days, where-

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as in spinal cord myelin there was an increase in protein from 8.73 per cent at 5 days to 18.85 per cent at 170 days.

In each of the foregoing investigations with laboratory animals, the greatest accumulation of protein took place during the general period of rapid myelination in the CNS.

Studies dealing with developing human brain myelin have indicated, however, that the concentration of total myelin protein remains rather constant during maturation. In an early paper, Eng and his co-workers (1968) reported that protein constituted approximately 24 per cent of the dry weight of myelin in humans ranging from the age of 10 weeks to 92 years, while recently, Fishman and his associates (1975) noted that protein constituted about 30 per cent of the dry weight of myelin in all of the stages of human development which they studied from birth to adulthood.

The general view of CNS myelin has been that its protein composition is rather simple, consisting of basic protein(s), proteolipid protein, and a high molecular weight protein designated as Wolfgram protein (Shooter and Einstein, 1971). Recently, however, there have been a number of reports of other minor protein components. A relatively low molecular weight protein which migrates between the proteolipid and basic protein has been observed in a variety of gel systems (Mehl and Halaris, 1970; Greenfield et al., 1971; Druse and Hogan, 1972; Fishman et al., 1975). Agrawal and his co-workers (1972) have designated this protein, DM-20, on the basis of polyacrylamide gel electrophoresis in sodium dodecyl sulphate. Some high molecular weight proteins have also been detected in highly purified myelin preparations (Mehl and Halaris, 1970; Greenfield et al., 1971; Morell et al., 1972). Two enzymes, 2'3'-cyclic nucleotide phosphohydrolase (Kurihara and Tsukada, 1967) and cholesterol

ester hydrolase (Eto and Suzuki, 1973) have been established to be myelin components. The former has been shown to be a high molecular weight protein (Braun and Barchi, 1972). Recent studies by Quarles and his associates (1972, 1973) have provided evidence that a glycoprotein also should be added to the group of high molecular weight proteins which are associated with myelin.

Certain groups of researchers have conducted investigations to determine whether different quantitative changes occur in the various protein components of CNS myelin during development. In a study with human brain myelin, Eng and his associates (1968) observed that the encephalitogenic basic protein increased from 16 to 28 per cent of the total myelin protein (dry weight) from infancy (10 weeks) to adulthood (41 years). In addition, they found that in the 10 week old, 4 year old, and adult human, the proteolipid protein accounted for 68, 60, and 53 per cent, respectively, of the total myelin protein, whereas in the same age groups, the Wolfgram protein accounted for 16, 19, and 19 per cent, respectively, of the total myelin protein.

In another more recent study with human brain myelin, Fishman and his co-workers (1975) reported that the amount of encephalitogenic basic protein increased from 8.5 per cent of the total myelin protein (dry weight) in the newborn to 24 per cent at 16 months of age, and thereafter increased to approximately 30 per cent in the adult. They observed that the concentration of both the proteolipid protein and the DM-20 protein appeared to increase during maturation as evidenced by the increasing densitometric peak size when equal amounts of total protein were subjected to sodium dodecyl sulphate electrophoresis.

These investigators also studied the specific activity of the

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enzyme, 2', 3'-cyclic nucleotide-3'-phosphohydrolase in human myelin. The activity of this enzyme, when expressed as micromoles of adenosine 2'-monophosphate produced per mg protein per hour, did not change significantly after the newborn period of development. This finding is similar to that reported by two other groups of researchers who found that the specific activity of this enzyme did not change at successive stages of development in rat brain myelin (Quarles et al., 1973; Druse et al., 1974).

Einstein and her associates (1970) investigated the protein composition of the rabbit brain during maturation and reported that the proteolipid protein increased from 7.4 to 18.8 per cent of the total brain protein (dry weight) during the period from 1 to 120 days of age. They stated that the steadily increased ratio of proteolipid protein to total protein was a reflection of myelogenesis.

These same researchers also conducted electrophoretic studies on myelin proteins from rabbits at four ages (days 1, 5, 9, and adult). They observed that the proteolipid protein and the encephalitogenic basic protein increased as myelination proceeded, and they stated that the former protein appeared at an earlier stage of development. This latter finding is contrary to data reported for myelin proteins in developing humans (Eng et al., 1968; Fishman et al., 1975), mice (Morell et al., 1972), and rats (Adams and Osborne, 1973), and probably reflects the inability of the electrophoretic technique used by Einstein and her collaborators (1970) to detect small concentrations of the basic protein during the early period of development.

Morell and his co-workers (1972) reported marked changes in the quantitative distribution of certain proteins in myelin isolated from the

brains of maturing mice. The proportion of total myelin protein (dry weight) which was represented by the sum of two encephalitogenic basic proteins increased from approximately 18 per cent at 8 days to 30 per cent at 300 days of age. During the same time interval, the proteolipid protein increased even more dramatically, from 7 to 27 per cent. The Wolfgram protein appeared to remain fairly constant, however, and accounted for about 12 per cent of the total myelin protein at all of the ages which were examined. These investigators also observed that the period of greatest increase in the percentage composition of the basic and proteolipid proteins occurred between 10 to 25 days of age, and they noted that the accumulation of proteolipid protein was greater than the sum of the two encephalitogenic basic proteins which resulted in a subsequent decrease in the ratio of basic to proteolipid protein from 2.25 at 10 days of age to 1.25 at 25 days and 1.08 at 300 days of age.

Different groups of investigators have reported findings concerning changes in the myelin proteins during maturation of the rat brain. Gaitonde and Martenson (1970) observed that the concentration of proteolipid protein in the whole rat brain increased until 20 days after birth (approximately 2 mg/g wet brain), whereas the concentration of total basic protein in the brain increased until 40 days after birth (approximately 3 mg/g wet brain). They stated that the two encephalitogenic basic proteins accounted for about 45-53 per cent of the total basic proteins in the adult brain, and they indicated that the increase in basic proteins after 10 days of age was due mainly to the accumulation of the two encephalitogenic proteins which together comprised 0.13 and 1.84 mg/g wet brain on the 15th and 55th day after birth, respectively.

In a later study dealing with maturation of the rat brain, Norton

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and Poduslo (1973) reported that the proteolipid protein increased from 0.67 to 2.18 per cent of the dry weight of the brain in animals ranging from 15 to 425 days of age.

Adams and Osborne (1973) investigated the protein composition of brain myelin isolated from rats between the ages of 5 to 60 days. They defined the myelin they obtained from 5 to 11 day old animals as 'early myelin'. The only major myelin specific protein that could be detected in this material was the larger encephalitogenic basic protein, which first appeared at 7 days after birth. They observed that a changeover from 'early myelin' to adult type myelin occurred between 11 and 12 days after birth. This change corresponded with the first appearance of the proteolipid protein and the smaller encephalitogenic basic protein.

The authors of this study reported that in 12 day old myelin, the combined sum of the two encephalitogenic basic proteins and the proteolipid protein accounted for 73 per cent of the total myelin protein, while other minor protein components constituted 27 per cent. The larger basic protein by itself comprised about 24 per cent of the total protein. In 60 day old myelin, the proportions were approximately 89 per cent and 11 per cent, respectively, while the proportion of the larger basic protein dropped to about 16 per cent.

During the 12 to 60 day period examined in the foregoing study, the proteolipid protein and the smaller basic protein increased in relative amounts to the larger basic protein. Thus, despite the proportional decrease in the latter protein, the total amount of the two basic proteins maintained a constant ratio to the proteolipid protein during the different stages of myelin maturation.

In 1973, Quarles and his associates reported that adult rat brain

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myelin contained a minor glycoprotein component which constituted about 0.3-2.0 per cent of the total myelin protein. Druse and her co-workers (1974) measured the amount of this glycoprotein by Schiff staining in developing rat brain myelin and found that its concentration per mg of total myelin protein remained nearly constant over the period from 14 to 60 days after birth.

Recently, Zgorzalewicz and his associates (1974) were able to isolate highly purified myelin fractions from the forebrain, cerebellum, and spinal cord of rats at different ages and examine the protein composition of these myelin fractions by sodium dodecyl sulphate polyacrylamide gel electrophoresis. They found that definite compositional differences appeared in the myelin proteins of the three CNS regions during development. In regard to the proteolipid protein, a slight increase was observed during the initial two weeks in the forebrain and during the initial seven weeks in the cerebellum, with no major change occurring thereafter. In both of these regions, the proteolipid protein accounted for approximately 37 per cent of the total myelin protein in 90 day old adult animals. There was no change during development, however, in the proportion of proteolipid protein in the spinal cord, and this protein consitituted about 33 per cent of the total myelin protein in this region.

Zgorzalewicz and his co-workers (1974) reported that the ratio of the smaller encephalitogenic basic protein to the larger encephalitogenic basic protein increased in each of the CNS regions examined, with the following ranges: from 0.76 (15 days) to 2.35 (365 days) in the forebrain; from 0.68 (10 days) to 1.99 (365 days) in the cerebellum; and from 0.66 (5 days) to 2.2 (365 days) in the spinal cord. The change in the ratio of these two basic proteins was most pronounced during the

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early phases of myelination. The ratio of the proteolipid protein to the sum of the two basic proteins remained essentially constant, however, during development: 0.769 for the forebrain; 0.702 for the cerebellum; and 0.598 for the spinal cord.

In the same study by these investigators, the relatively low molecular weight DM-20 protein underwent no major change in the forebrain during myelin maturation, and it represented approximately 7 per cent of the total myelin protein in this region. A small consistent decrease of this protein was observed, however, in the cerebellum and in the spinal cord. During a time period ranging from 10 to 90 days in the former region and 5 to 90 days in the latter region, the DM-20 protein declined from approximately 11 to 5 per cent of the total myelin protein.

Zgorzalewicz and his associates (1974) noted that the high molecular weight Wolfgram protein showed a marked decrease in the forebrain, where it dropped from about 16 per cent of the total myelin protein at 15 days to about 6 per cent at 90 days. The decrease in this protein was much less pronounced in the cerebellum and in the spinal cord. In the former region, it fell from about 10 per cent of the total myelin protein at 15 days to about 6 per cent at 90 days, while in the latter region, it declined from approximately 9 per cent at 5 days to 6 per cent at 90 days. The decrease of the Wolfgram protein during development is in accord with the idea that it may serve as a transitory protein in the formation of compact myelin from the oligodendroglial membranes (Waehneldt and Neuhoff, 1974), possibly as part of the myelin-like fraction which is present in immature CNS tissue (Agrawal et al., 1970).

The finding in the present investigation that there is an increase in the total protein content in brain myelin of maturing Long-Evans rats

is in agreement with the data previously discussed from other studies with small laboratory animals. In most of these investigations, including the current one, myelin was isolated from whole brains and, therefore, the accumulation of myelin protein during development must be considered as the sum of different developmental stages in various regions of the brain.

The type of ontogenetic study conducted by Zgorzalewicz and his associates (1974), involving the protein composition of myelin isolated from separate CNS areas, has emphasized that marked differences occur during maturation with respect to the amount of myelin protein which accumulates in various regions and particularly in regard to proportional modifications in the specific myelin proteins.

In order to acquire more accurate information about the ontogenetic development of myelin proteins, future experiments should be carried out on myelin isolated from separate CNS regions rather than whole CNS tissue. These future studies should include electrophoretic densitometric measurements of the proteins in both myelin and myelin-like fractions from various species at very early developmental stages. In addition, amino acid labelling techniques might be utilized to determine the rates and times of synthesis of the individual myelin proteins which are being formed during the process of maturation.

II. Histological Studies

A. Experimental Allergic Encephalomyelitis (EAE) Studies.

In the present series of investigations, Long-Evans rats were sensitized with complete Freund's adjuvant containing two basic proteins from rat brain myelin, one basic protein from bovine spinal cord myelin, one

basic protein from guinea pig spinal cord myelin, or whole wet guinea pig spinal cord.

The findings from these investigations, which have shown this strain of rats is not highly susceptible to the induction of EAE, confirm and extend the information from an earlier study by Levine and Wenk (1961), who reported that Long-Evans hooded rats from Rockland Farms were less susceptible to the induction of EAE than Sherman, HH Wistar, and CFN rats after sensitization with complete Freund's adjuvant containing 120 mg of whole rat spinal cord.

In the present study, flaccid paralysis of the hind legs, which is a characteristic clinical sign of EAE, was observed in only 4 out of the 54 experimental rats. Two of the animals became paralyzed on the 12th and 13th day after a single injection of 120 µg of guinea pig basic protein in 0.1 ml complete Freund's adjuvant. Paralysis occurred in the other 2 animals on the 12th and 16th day after a single injection of 8,000 µg of whole wet guinea pig spinal cord in 0.2 ml complete Freund's adjuvant.

A weight loss of 17 to 24 grams preceded the appearance of paralysis in these animals. This early loss of weight is a common occurrence in animals affected with EAE.

All of the experimental rats had very active inoculation sites in the foot pads which showed progressive enlargement, inflammation, ulceration, and crusting, followed by partial or complete healing. Levine and Wenk (1961) reported that the Long-Evans rats in their study had inoculation sites which were as active as the sites found in the Sherman and HH Wistar rats which were highly susceptible to EAE, whereas the CFN and Sprague-Dawley rats, which demonstrated a low susceptibility to EAE,

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exhibited much less active inoculation sites. They concluded that the Long-Evans strain was somewhat anomalous in showing low susceptibility to EAE despite strongly reactive inoculation sites.

In the present investigation, EAE lesions were demonstrable in the CNS of clinically asymptomatic rats as well as in those with paralysis. CNS lesions were present in at least one rat in each experimental group which was sensitized with complete Freund's adjuvant containing various amounts of rat basic proteins, guinea pig basic protein, or whole guinea pig spinal cord. There was no evidence, however, of any EAE lesions in the group of experimental animals which were sensitized with bovine basic protein in complete Freund's adjuvant.

Guinea pig neuroantigens appeared to be the most effective agents for inducing EAE in the present work with Long-Evans rats. They produced EAE lesions in the greatest number of animals, in addition to being the only neuroantigens which caused clinical evidence of hind leg paralysis. Out of the three types of neuroantigens which were tested and produced EAE lesions, whole guinea pig spinal cord was more effective than guinea pig basic protein, which in turn, was more effective than the two rat basic proteins.

The microscopic details of the EAE lesions in the Long-Evans rats were similar to those described for other strains of rats (Levine and Wenk, 1961; Paterson, 1966; Levine, 1974). The lesions consisted of vascular and perivascular accumulations of mononuclear inflammatory cells which spread into the parenchyma of the CNS for varying distances. These cells, which were pleomorphic, resembled large and small lymphocytes and monocytes, with lymphocytes being the more prevalent cell type.

There was variability in the location, number, and size of the CNS

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lesions in the rats affected with EAE. Lesions were present at various levels of the neuroaxis, but there was a predilection for larger and more numerous lesions in the spinal cord and brain stem (medulla and lower pons). All of the 13 EAE affected animals had lesions in these two general areas, whereas 7 had lesions in the cerebellum, and only 4 had lesions in the cerebrum. The lesions in the latter two regions tended to be small and few in number. Other investigators have also reported a predilection for EAE lesions in the spinal cord, medulla, and cerebellum (decreasing order) of various rat strains (Levine and Wenk, 1961; Levine, 1974). The specific areas of the CNS affected by EAE, however, vary with different species (Boehme et al., 1974). Regional differences in the chemical composition of CNS structures might be one of several factors which contribute to this phenomenon.

In most species, EAE lesions are found in both the gray and white matter of the CNS, but there is a predilection for the latter (Levine, 1971). This was the case in the present study. In the small number of animals with hind leg paralysis, there was a tendency for EAE lesions to occur in both the white and gray matter, but in the majority of animals, the lesions were distributed in the white matter. Vascular and perivascular infiltrations of mononuclear inflammatory cells were especially apt to be found in the long tracts of the spinal cord white matter and near the area of the fourth ventricle. Foci of inflammatory cells were also frequently present in and beneath the meninges surrounding the spinal cord.

Demyelination during EAE is more prominent in large animals than in small laboratory animals which tend to show perivascular changes rather than the typical picture of demyelination (Boehme et al., 1974;

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Hirsch and Parks, 1975). Recently, Smith and her associates (1974) reported that in rats with EAE the loss of myelin is so small that it is barely detectable. In the present study, there was also no appreciable injury to myelin in the rats affected with EAE.

Striking differences in susceptibility to EAE have been reported for the various species which have been most commonly used in EAE investigations (Kies, 1973). In addition, different ranges of susceptibility have been observed among individual monkeys and rabbits (Kies, 1973), as well as among different strains of guinea pigs (Stone, 1962; Stone et al., 1969; Kies et al., 1975; Lisak et al., 1975), mice (Olitsky et al., 1950; Levine and Sowinski, 1973; Bernard and Carnegie, 1975), and rats (Levine and Wenk, 1961, 1965a; Kornblum, 1968; Hughes and Stedronska, 1973; Graham et al., 1974; McFarlin et al., 1975).

In the present study, Long-Evans rats were more susceptible to the induction of EAE when they were sensitized with whole guinea pig spinal cord than when they were sensitized with guinea pig, rat, or bovine myelin basic proteins.

Other investigations of EAE in rats have shown that in certain strains, whole guinea pig spinal cord is highly encephalitogenic (Levine and Wenk, 1961, 1965a; Paterson et al., 1970; Smith et al., 1974), whereas in other strains, it is only weakly encephalitogenic (Hughes and Stedronska, 1973; Levine and Sowinski, 1975).

In Lewis rats, which are a strain highly susceptible to EAE, Paterson and his associates (1970) found that whole guinea pig spinal cord (xenogeneic tissue) was more encephalitogenic than allogeneic spinal cord tissue from Wistar rats; the latter tissue, in turn, was more active than syngeneic spinal cord tissue from Lewis rats. These

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findings were not due merely to the degree of foreigness of the sensitizing material, because other xenogeneic spinal cord tissue (bovine) was less active than the foregoing tissues (Levine and Wenk, 1965a).

Differences between the encephalitogenicity of whole spinal cord tissue and of purified myelin basic proteins have also been noted in certain strains of rats and guinea pigs. Smith and her associates (1974) found that the Wistar strain of rats in their laboratory, which were not highly susceptible to EAE, would respond only to sensitization with whole guinea pig spinal cord, and not to guinea pig myelin basic protein. Levine and Sowinski (1975) reported that in the relatively EAE resistant strain of Brown Norway rats, they were able to produce severe EAE with whole rat spinal cord in carbonyl iron adjuvant and weak EAE with whole guinea pig spinal cord. They were unable to produce any evidence of EAE in this rat strain, however, with a variety of doses of myelin basic proteins from either rats or guinea pigs. Kies and her co-workers (1975) observed that whole guinea pig spinal cord was more encephalitogenic than guinea pig myelin basic protein in Strain 2 guinea pigs which are known to be less susceptible to EAE than Strain 13 and Hartley guinea pigs (Lisak et al., 1975).

The differences between the encephalitogenicity of whole spinal cord tissue and myelin basic protein(s) are puzzling because no purified CNS component other than the basic proteins(s) has been discovered to be encephalitogenic.

Lewis rats, which are highly susceptible to EAE, have been the strain of rats most commonly used to test the encephalitogenic activities of myelin basic proteins and to identify the encephalitogenic determinants in the peptide fragments derived from them.

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Studies carried out with intact myelin proteins in this rat strain have shown that the severity of EAE is related to the species origin of the basic protein used. Kies and her associates (1972) and Martenson and his co-workers (1972a) found that both the larger and smaller basic proteins from syngeneic and allogeneic strains were highly active at levels of 10 µg. Activity was marginal or absent at 2 µg levels. Guinea pig basic protein was the most active protein tested, inducing severe EAE at a level as low as 2 µg. Bovine, rabbit, monkey, and human basic proteins were much less active than guinea pig and rat proteins. Fifty µg quantities of the former group of proteins were required to produce mild EAE. Chicken and turtle proteins were approximately as active as the bovine protein at levels of 50 µg, whereas the frog protein appeared to be less active. Fifty µg of carp and shark basic proteins did not induce any clinical or histological evidence of EAE.

In a later study with Lewis rats, McFarlin and his associates (1973) reported that guinea pig basic protein was more encephalitogenic that the smaller rat protein from syngeneic and allogeneic strains, and bovine basic protein was the least active of the proteins.

The foregoing investigations suggested that differences in the amino acid sequences in the encephalitogenic regions of the basic protein molecules might account for variations in their activity in Lewis rats.

In 1973, Dunkley and his co-workers isolated a peptide fragment from the smaller rat basic protein (residues 45-86) which was equally as encephalitogenic as the intact protein in Lewis rats.

In another study, McFarlin and his associates (1973) isolated

peptides containing 45 residues (residues 44-89) from guinea pig, bovine, and the smaller rat basic proteins. When they tested the activity of these peptides in Lewis rats, they found that the guinea pig peptide was as highly encephalitogenic as the intact protein. The activity of the rat peptide was also comparable to that of the intact protein and, like the latter, it was less encephalitogenic than the guinea pig peptide. The bovine peptide was inactive, in contrast to the intact protein, which was mildly encephalitogenic.

These investigators suggested that the variations in the encephalitogenic activity of the peptides might be due to different amino acid sequences which they found at the N- and C-terminal ends of the peptides. In addition, they pointed out that since the intact bovine basic protein was mildly active, this molecule might contain other encephalitogenic regions which are different from the 45-residue peptide they studied.

Recently, Martenson and his co-workers (1975c) conducted further tests on regions of the bovine and guinea pig basic proteins which are encephalitogenic in Lewis rats. They found that the weakly encephalitogenic bovine protein has at least two mutually exclusive encephalitogenic regions. The more active region, which is located within the N-terminal half of the protein, includes the sequence Asp-Ser-Leu-Gly-Arg-Phe (residues 37-42). The less active region is located within the C-terminal half of the protein. It begins between residues 88 and 111 and ends before residue 153. This region contains the sequence Leu-Ser-Leu-Ser-Arg-Phe (residues 108-113), which is very similar to the first encephalitogenic determinant.

Studies by Martenson and his associates (1975c) on the extremely encephalitogenic guinea pig protein showed that virtually all of its

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activity could be recovered in the peptides containing residues 37-88 and 43-88. Their data suggested that a weaker determinant might be also present in the guinea pig peptide containing residues 89-169, since this peptide was found to have the same activity as the corresponding bovine peptide.

Variations with respect to the determinants responsible for the induction of EAE have been observed in other species besides rats. Kies (1973) and Martenson and his associates (1975b) have reviewed the findings concerning the different sites which have been found to be encephalitogenic in certain species. These variations are not well understood at the present time, but they may reflect genetic and immunologic specificities.

Recently, genetic studies have been conducted whereby rat strains which are highly susceptible to EAE were crossed with rat strains which are less susceptible to the disease.

The findings from two investigations, in which (HS) Lewis and (LS) Brown Norway rats were crossed and the progeny sensitized with whole guinea pig spinal cord (Gasser et al., 1973) or guinea pig basic protein (Williams and Moore, 1973) in complete Freund's adjuvant, suggested that susceptibility to EAE might be largely determined by a single dominant gene which is closely linked to the major Ag-B histocompatability locus. It was proposed that this is an immune response gene, designated Ir-EAE, which controls the reactivity of T-cells (thymus-derived lymphocytes) directed against the encephalitogenic determinants in the basic protein molecule.

Recent immunological studies with Lewis and Brown Norway rats have provided evidence which supports this concept that an immune response

gene(s) is involved with the cell-mediated response in EAE (McFarlin et al., 1975).

The single dominant gene hypothesis requires that the (L x BN) F_1 hybrids should be as reactive as the Lewis strain. Recently, Levine and Sowinski (1975) reported that data concerning the incidence and severity of the clinical and pathological signs of EAE indicated the (L x BN) F_1 hybrids were intermediate in reactivity between the two parental strains.

In another study, Gasser and his associates (1975) observed that when (LS) Brown Norway rats were crossed with the DA strain, which is quite susceptible to EAE, the (DA x BN) F_1 hybrids were less susceptible to EAE than the (L x BN) F_1 hybrids. In addition, when Lewis rats were crossed with the resistant AVN strain, almost all of the (L x AVN) F_1 hybrids were resistant to EAE (Perlik and Zidek, 1974).

The foregoing findings would appear to indicate that EAE susceptibility may involve more complex modes of genetic inheritance than a single dominant (Ir-EAE) gene. Perhaps there are modifying genes which also affect the different patterns of EAE responses in animal strains.

In light of the information which has been presented, the relatively low susceptiblity of Long-Evans rats to EAE may be due to inherent genetic factors.

Future work with Long-Evans rats should include genetic experiments in order to evaluate the mode of inheritance involved in EAE susceptibility in these animals. Immunological studies should also be carried out in order to determine the capacity of these animals to mount immune responses. In addition, more extensive EAE investiga-

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tions should be conducted with this rat strain in order to test the effects of different types and amounts of adjuvants and encephalitogenic neuroantigens.

B. Encephalitogenic Tolerance Studies.

Two studies have indicated that sensitization of newborn Wistar rats with whole guinea pig, rabbit, or rat spinal cord in saline will result in a decreased capacity of these animals to develop EAE lesions when tested at adult ages (Paterson, 1958; Waksman, 1959b).

The findings from these studies led to the hypothesis that the adult animal's ability to develop EAE depends on its failure to acquire tolerance to the myelin antigen, the antigen being in such small amounts during the early period of development that it fails to affect the immune apparatus. In view of this hypothesis, a series of experiments were undertaken to investigate whether Long-Evans rats, the offspring of mothers sensitized with the two specific rat myelin basic proteins, would show a decreased ability to develop EAE lesions when tested at an adult age.

In the present study, maternal sensitization with 43.2 μg of rat basic proteins in incomplete Freund's adjuvant on either the 4th day (preimplantation) or 8th day (postimplantation) of pregnancy did not appear to decrease the capacity of the adult offspring to develop EAE lesions when they were sensitized with various amounts of rat basic proteins or whole guinea pig spinal cord in complete Freund's adjuvant.

There was no significant reduction in the number of offspring with EAE lesions. A slightly larger ratio of the offspring had EAE lesions after sensitization with whole guinea pig spinal cord than after sensi-

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tization with rat basic proteins which concurs with the findings in the EAE Studies (Section A).

There was no apparent decrease in the severity of the EAE lesions in the affected offspring. The lesions showed the same basic characteristics and the same patterns of distribution as those described in the EAE Studies (Section A). Luxol fast blue stains of CNS sections from the affected offspring did not reveal any appreciable injury to myelin which also concurs with the findings in the EAE Studies (Section A).

None of the offspring with EAE lesions showed evidence of hind leg paralysis. This cannot be considered a significant finding, however, in view of the fact that Long-Evans rats rarely demonstrate this clinical symptom of EAE.

The inability to induce tolerance in the present study may be due to the fact that the neuroantigen failed to affect the immune apparatus because it was given too early during pregnancy, it was given in too low an amount, or it did not cross the placenta effectively. Therefore, in future experiments, the animals should be sensitized at later periods during pregnancy with larger amounts of encephalitogenic myelin basic proteins or peptides in the form of single and multiple injections. In addition, future experiments of this type should also be conducted on other strains of rats and on other species which are highly susceptible to EAE.

C. Fetal Studies.

Three separate studies using mice have shown that different CNS defects may be induced in embryos as a result of maternal sensitization (prior to breeding) with complete Freund's adjuvant containing whole

brain tissue from mice (Gluecksohn-Waelsch, 1957; Barber et al., 1959, 1961) or rabbits (Barber et al., 1961). The investigators suggested that the CNS abnormalities observed in their experiments ultimately might be the result of specific or non-specific antibody responses.

A complex number of variables may have contributed to the teratogenic effects in the foregoing studies because the mice were sensitized with a very non-specific type of neuroantigen which was administered in the form of multiple injections over broad time periods prior to breeding.

In order to reduce the number of variables in this type of teratogenic experiment, the present study with Long-Evans rats consisted of maternal sensitization with a specific neuroantigen (two rat myelin basic proteins) on the 4th day (preimplantation) or 8th day (postimplantation) of pregnancy.

Maternal sensitization with 131.2 μg of rat myelin basic proteins in incomplete Freund's adjuvant on either the 4th or 8th day of pregnancy did not result in any gross or internal abnormalities in the 125 experimental fetuses which were examined at 21 days of age. There was also no histological evidence of abnormalities in the development of the skull, brain, eyes, ears, mouth, and nose in the 12 experimental fetal heads which were serially sectioned and stained with hematoxylin and eosin.

In the present investigation, the neuroantigen was administered to the pregnant rats in incomplete Freund's adjuvant in order to prevent paralysis from occurring in these animals. Since it is now known that Long-Evans rats rarely develop paralysis during EAE, it would seem

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reasonable to repeat this experiment using larger amounts of neuroantigen in combination with complete Freund's adjuvant and other types of adjuvants which are known to facilitate the induction of EAE.

Future experiments of this type should also be conducted on other strains of rats and on other species which are highly susceptible to EAE in order to obtain more conclusive information as to the possible teratogenic activity of encephalitogenic myelin basic proteins and peptides. The animals in these future studies should be sensitized with different amounts of specific neuroantigens in order to determine the lowest levels at which they may exert teratogenic effects. In addition, the animals should also be sensitized on different days before breeding and during pregnancy in order to determine whether the neuroantigens require a long or short time period to produce teratogenic effects.

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SUMMARY AND CONCLUSIONS

Myelin basic proteins from the CNS of Long-Evans rats were studied in regard to their development with age and their ability to produce EAE, protect against EAE, and induce teratogenic effects.

Polyacrylamide disc electrophoresis studies of basic protein extracts (pH 1.8) from the CNS myelin of mature and immature Long-Evans rats showed that two basic protein components were present in the CNS myelin of this strain of rats.

The larger basic protein, which had a slower electrophoretic mobility, was comparable to the single encephalitogenic basic protein found in guinea pig, rabbit, bovine, monkey, and human CNS myelin. The smaller basic protein, which had a faster cathodic mobility, was comparable to the additional encephalitogenic basic protein found in the CNS myelin of certain species of rodents.

The electrophoretic analyses of basic proteins extracted at pH 1.8 from myelin isolated from the brains of rats ranging in age from 14 to 130 days showed that both the larger and smaller encephalitogenic basic proteins were present as single, distinct electrophoretic bands as early as 14 days after birth.

Similar electrophoretic analyses of basic proteins extracted at pH 1.8 from whole brains of rats ranging in age from 1 to 130 days of age suggested that the two encephalitogenic basic proteins were probably present earlier than 14 days after birth. A faint electrophoretic band which appeared to correspond with the larger basic protein was detectable in the 1 day old brain samples. Two faint electrophoretic bands, which were very close to each other, were observed in the region of the smaller

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basic protein in the 1 and 5 day old brain samples. At 7 days after birth, these two protein bands appeared to merge into a single band which corresponded with the smaller basic protein found in the mature rat brain.

The different electrophoretic staining intensities of the two encephalitogenic basic proteins in both the myelin and whole brain studies indicated that proportionally there was a greater increase in the smaller basic protein than in the larger basic protein during brain maturation. In addition, the whole brain studies also showed that the increase in the two encephalitogenic basic proteins appeared to coincide with the reduction of other basic proteins in the maturing brain.

The investigations concerning the total protein content of brain myelin in maturing Long-Evans rats showed that myelin protein (per cent dry weight) increased from 7.87 per cent at 10 days to 23.17 per cent at 130 days. The period of greatest accumulation of myelin protein appeared to occur from 10 to 30 days after birth. This time period coincides with that of rapid myelination in the CNS of rats.

The experimental efforts to induce EAE in Long-Evans rats sensitized with complete Freund's adjuvant containing various amounts of rat, bovine, or guinea pig neuroantigens indicated that this strain of rats was not highly susceptible to the induction of EAE. Out of the 54 experimental animals, only 4 demonstrated clinical evidence of hind leg paralysis, and only 13 showed pathological evidence of EAE lesions.

Guinea pig neuroantigens appeared to be the most effective agents for inducing EAE in this strain of rats. They produced EAE lesions in the greatest number of animals, in addition to being the only neuroantigens which caused clinical evidence of hind leg paralysis. Out of the

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three types of neuroantigens which produced EAE lesions, whole guinea pig spinal cord was more effective than guinea pig myelin basic protein, which in turn, was more effective than the two rat myelin basic proteins. Bovine myelin basic protein was not effective in producing any EAE lesions.

The microscopic details of the EAE lesions were similar to those described for other strains of rats. The lesions consisted of vascular and perivascular accumulations of mononuclear inflammatory cells which spread into the CNS parenchyma. The mononuclear cells resembled large and small lymphocytes and monocytes, with lymphocytes being the prevalent cell type.

The EAE lesions were more commonly found in the CNS white matter than in the gray matter and there was a predilection for larger and more numerous lesions in the spinal cord and brain stem (medulla and lower pons). There was not any appreciable injury to myelin in the rats affected with EAE.

The relatively low susceptibility of Long-Evans rats to EAE may be due to genetic factors. Future work with this strain of rats should include genetic experiments in order to evaluate the mode of inheritance involved in EAE susceptibility in these animals. In addition, immunological studies should also be carried out in order to determine the capacity of these animals to mount immune responses.

The experimental efforts to induce tolerance to encephalitogenic neuroantigens in the offspring of Long-Evans rats were not successful. Maternal sensitization with 43.2 μg of rat myelin basic proteins in incomplete Freund's adjuvant on either the 4th or 8th day of pregnancy did not appear to decrease the capacity of the adult offspring to develop EAE lesions when they were sensitized with various amounts of

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rat myelin basic proteins or whole guinea pig spinal cord in complete Freund's adjuvant.

The inability to induce tolerance in the present study may be due to the fact that the neuroantigen failed to affect the immune apparatus because it was given too early during pregnancy, it was given in too low an amount, or it did not cross the placenta effectively. Therefore, in future experiments, the animals should be sensitized at later periods during pregnancy with larger amounts of encephalitogenic myelin basic proteins or peptides in the form of single and multiple injections. In addition, future experiments of this type should also be conducted on other strains of rats and on other species which are highly susceptible to EAE.

The encephalitogenic rat neuroantigen did not produce teratogenic effects in fetal Long-Evans rats. Maternal sensitization with 131.2 µg of rat myelin basic proteins in incomplete Freund's adjuvant on either the 4th or 8th day of pregnancy did not cause any gross or internal abnormalities in 21 day old fetuses. There was also no histological evidence of CNS abnormalities in the fetuses.

The absence of teratogenic effects in the present study may be related to the particular days of pregnancy chosen for sensitization or the amount and type of neuroantigen and adjuvant used for sensitization. Therefore, in future experiments, the animals should be sensitized on different days before breeding and during pregnancy with various amounts of encephalitogenic myelin basic proteins or peptides in combination with adjuvants which facilitate the induction of EAE. In addition, future experiments of this type should also be conducted on other strains of rats and on other species which are highly susceptible to EAE.

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FIGURES

ELECTRON PHOTOMICROGRAPHS

All of the myelin preparations in the 6 electron photomicrographs were isolated from the brains of postnatal Long-Evans rats. They were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate and postfixed in osmium tetroxide, dehydrated in a graded series of alcohols and propylene oxide, and embedded in Epon 812. The sections were cut on a LKB ultratome, mounted on naked grids, and stained with uranyl acetate and Reynold's lead citrate.

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FIGURE 1 A.

Myelin from 110 day old rats.

A low power micrograph showing many relatively thick multilamellar myelin forms. Note the absence of other recognizable subcellular structures. X6,000.

A

FIGURE 1 B.

Myelin from 110 day old rats.

A high power micrograph of a portion of Figure 1 A showing the characteristic multilamellar structure of a piece of myelin in which the major dense and intraperiod lines are visible.

X40,000.

B

FIGURE 2 A.

Myelin from 14 day old rats.

A low power micrograph showing both relatively thick and thin lamellar myelin forms. There are fewer thick pieces of myelin in comparison with Figure 1 A. Note the absence of other recognizable subcellular structures.

X6,000.

A

FIGURE 2 B.

Myelin from 14 day old rats.

A high power micrograph of a portion of Figure 2 A showing the major dense and intraperiod lines in a multilamellar piece of myelin.

X40,000.

B

UMI

FIGURE 3 A.

Myelin from 9 day old rats.

A low power micrograph showing some relatively thick lamellar myelin forms and many thin lamellar myelin segments. There are very few thick pieces of myelin in comparison with Figure 1 A. Note the absence of other recognizable subcellular structures.

X10,000.

A

FIGURE 3 B.

Myelin from 9 day old rats.

A high power micrograph of a portion of Figure 3 A showing lamellar myelin segments around a piece of myelin in which the major dense and intraperiod lines are visible.

X38,000.

B

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ELECTROPHORETIC PHOTOGRAPHS

All of the basic protein samples in the 6 electrophoretic photographs were extracted with HCl at pH 1.8 from myelin or whole brains of postnatal animals. They were assayed by the polyacrylamide disc electrophoretic method of Reisfeld, Lewis, and Williams (1962) for a pH 4.3, 15% gel system.

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FIGURE 4.

Electrophoretic distribution of encephalitogenic myelin basic proteins from a pooled number of rat brains and bovine spinal cords.

A. Rat, 130 days, 100 µg.

B. Bovine, adult, 50 µg.

KEY: LBP = Larger Myelin Basic Protein.

SBP = Smaller Myelin Basic Protein.

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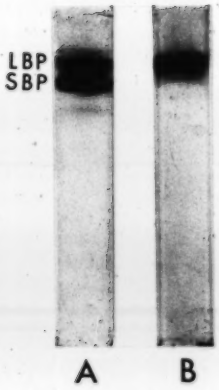
LBP
SBP
A
B

FIGURE 5.

Electrophoretic distribution of the two encephalitogenic myelin basic proteins from single brains of immature and mature rats.

A. 24 days, 200 µg.

B. 24 days, 300 µg.

C. 130 days, 200 µg.

KEY: LBP = Larger Myelin Basic Protein.

SBP = Smaller Myelin Basic Protein.

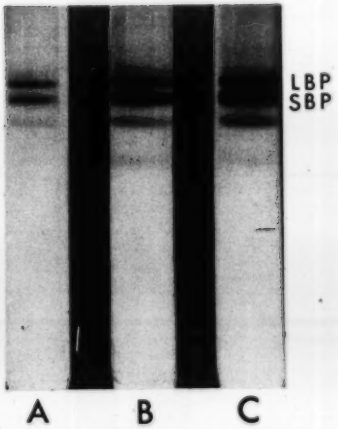
LBP
SBP
A
B
C

FIGURE 6.

Electrophoretic distribution of the two encephalitogenic myelin basic proteins from brains of rats at various stages of development. Myelin was isolated from a pooled number of rat brains.

A. 14 days, 300 µg.

B. 16 days, 100 µg.

C. 18 days, 100 µg.

D. 20 days, 100 µg.

E. 24 days, 100 µg.

F. 30 days, 100 µg.

G. 50 days, 100 µg.

H. 70 days, 100 µg.

I. 90 days, 100 µg.

J. 110 days, 100 µg.

K. 130 days, 100 µg. (The two dense bands were actually separate although they appear to be united in the picture.)

KEY: LBP = Larger Myelin Basic Protein.

SBP = Smaller Myelin Basic Protein.

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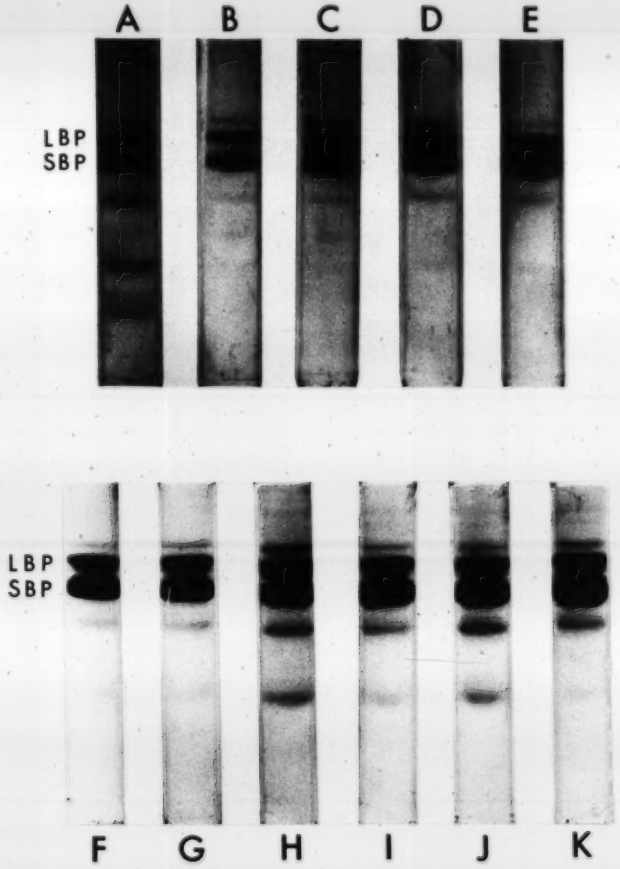

A
B
C
D
E
LBP
SBP
LBP
SBP
F
G
H
I
J
K

FIGURE 7.

Electrophoretic distribution of basic proteins from single brains of rats at various stages of development.

A. 1 day, 200 µg.

B. 5 day, 200 µg.

C. 7 days, 200 µg.

D. 9 days, 200 µg.

E. 12 days, 200 µg.

F. 130 days, encephalitogenic myelin basic proteins for comparison, 200 µg.

G. 14 days, 200 µg.

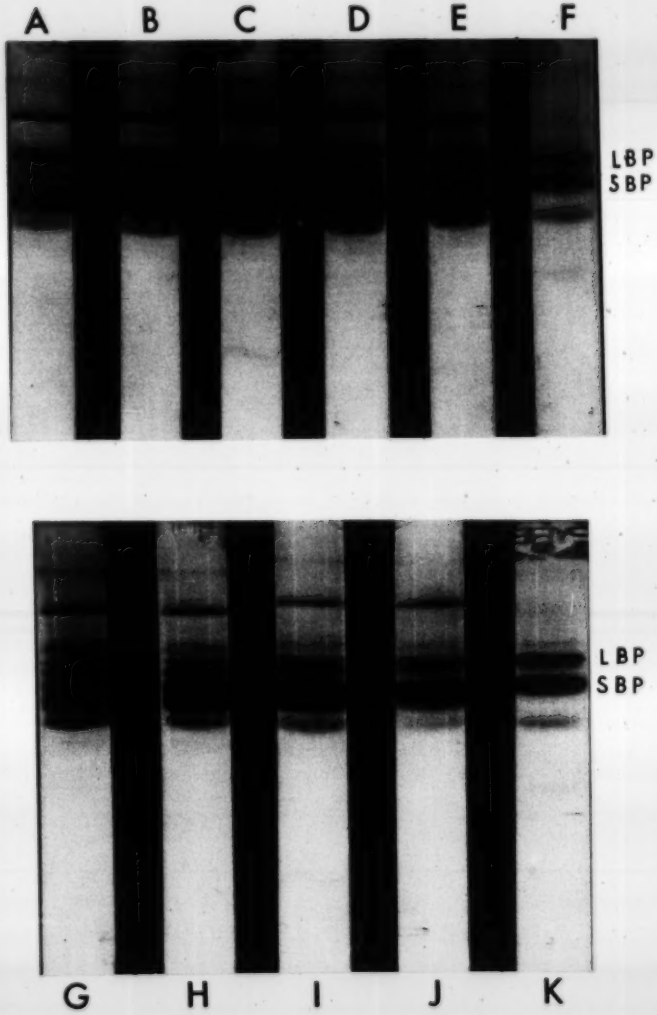
H. 18 days, 200 µg.

I. 24 days, 200 µg.

J. 130 days, 200 µg.

K. 130 days, encephalitogenic myelin basic proteins for comparison, 200 µg.

KEY: LBP = Larger Myelin Basic Protein.
SBP = Smaller Myelin Basic Protein.


A
B
C
D
E
F
LBP
SBP
LBP
SBP
G
H
I
J
K

HISTOLOGICAL PHOTOMICROGRAPHS

All of the CNS samples in the 24 histological photomicrographs were from adult Long-Evans rats. They were fixed in neutral buffered 10% formalin, dehydrated in a graded series of alcohols, cleared in chloroform, and infiltrated and embedded in paraffin (M.P. 56-58^{o}C). The sections were cut at 10 microns and stained with Harris hematoxylin and eosin.

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FIGURE 8.

EAE lesions in the CNS of a rat sensitized with 8,000 µg of whole guinea pig spinal cord in complete Freund's adjuvant.

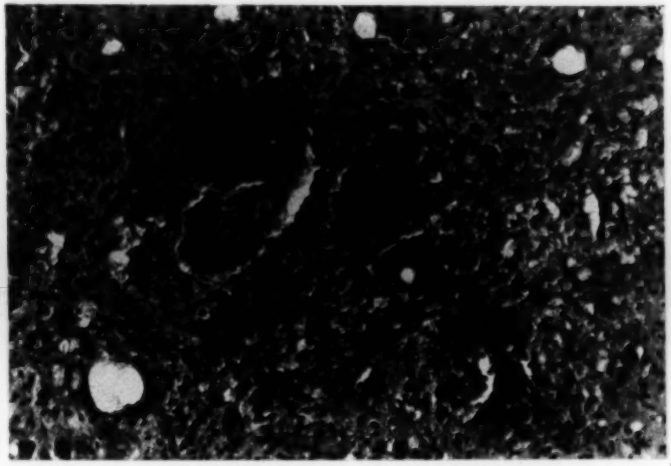
A. Vascular and perivascular infiltrations of mononuclear inflammatory cells in the medulla near the fourth ventricle. X203.

B. Higher magnification of the central lesion in Figure A. X354.

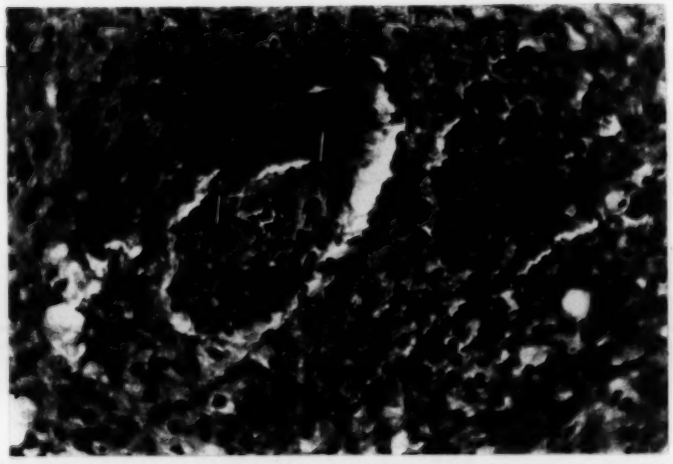
C. Higher magnification of Figure B. Note that most of the mononuclear inflammatory cells resemble lymphocytes. X728.

UMI

A



B



C

FIGURE 9.

EAE lesions in the CNS of a rat sensitized with 8,000 µg of whole guinea pig spinal cord in complete Freund's adjuvant.

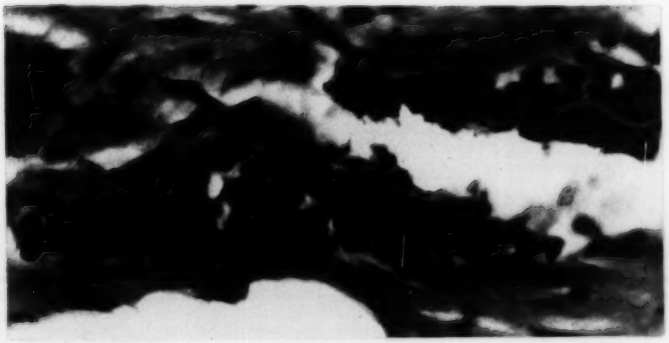
A. Two focal areas of mononuclear inflammatory cells in the white matter of the spinal cord.
X203.

B. Mononuclear inflammatory cells in a small focal area of the optic tract in the cerebrum.
X728.

C. Higher magnification of Figure B. Note that most of the cells resemble lymphocytes.
X1732.

A

B



C

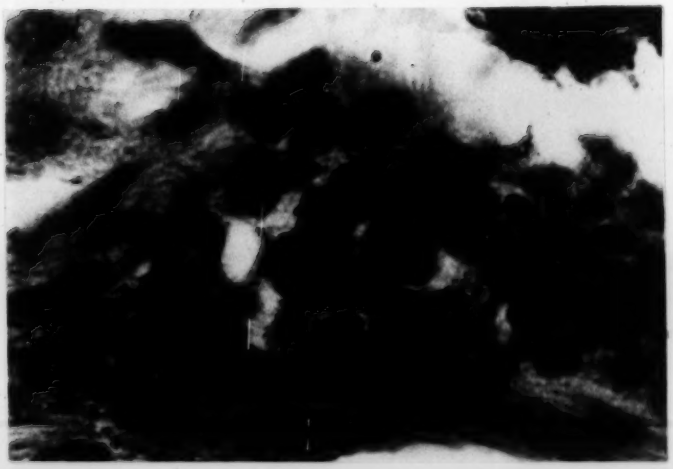


FIGURE 10.

EAE lesions in the CNS of a rat sensitized with 120 µg of guinea pig myelin basic protein in complete Freund's adjuvant.

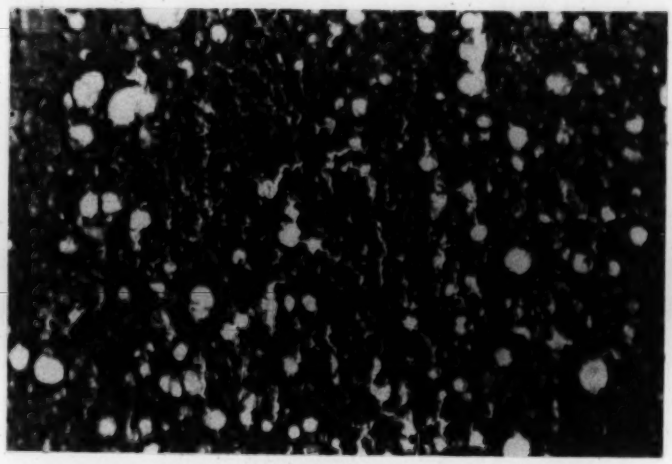
A. Diffuse infiltrations of mononuclear inflammatory cells in the white matter of the spinal cord. Note that some of the cells are entering the gray matter at the base of the photograph. X192.

B. Vascular and perivascular infiltrations of mononuclear inflammatory cells in a small region of the internal capsule in the cerebrum. X192.

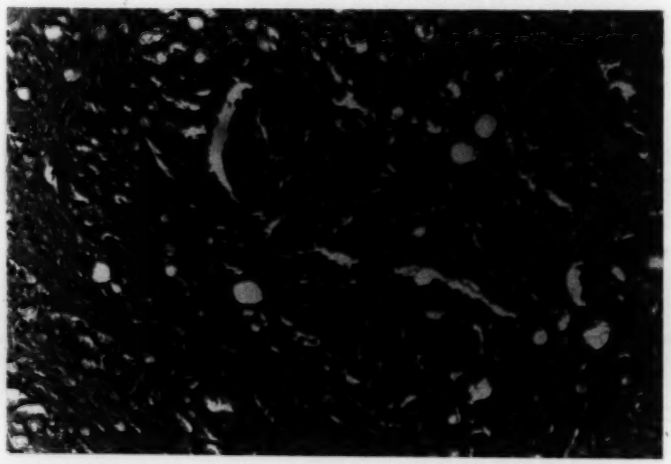
C. Higher magnification of Figure B. Note that most of the cells resemble lymphocytes. X728.

UMI

A



B



C

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FIGURE 11.

EAE lesions in the CNS of a rat sensitized with 240 µg of guinea pig myelin basic protein in complete Freund's adjuvant.

A. Vascular and perivascular infiltrations of mononuclear inflammatory cells in the medulla near the region of the fourth ventricle. X192.

B. Higher magnification of the central lesion in Figure A. X728.

A

B

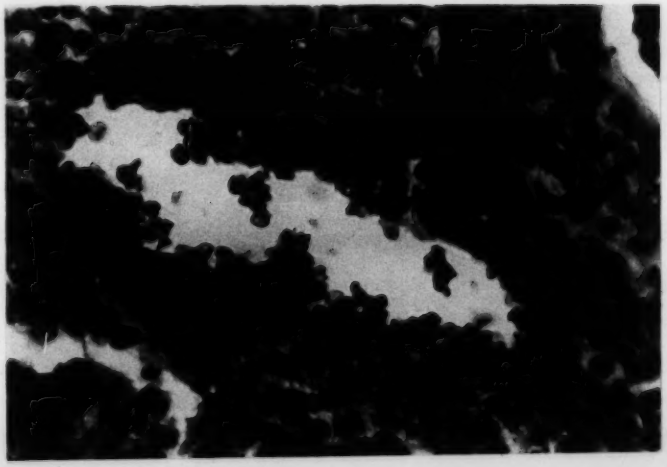

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FIGURE 12.

EAE lesions in the CNS of rats sensitized with 43.2 µg (A), 131.2 µg (B), and 262.4 µg (C) of rat myelin basic proteins in complete Freund's adjuvant.

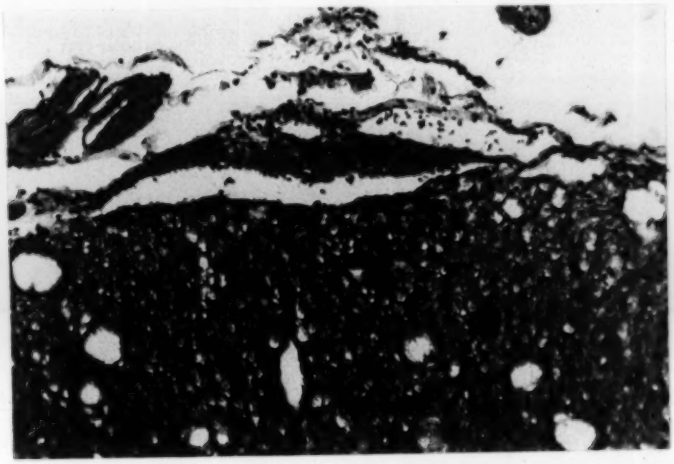
A. Mononuclear inflammatory cells in the meninges and in the white matter near the edge of the medulla.
X187.

B. A narrow focal area of mononuclear inflammatory cells in the white matter of the spinal cord.
X187.

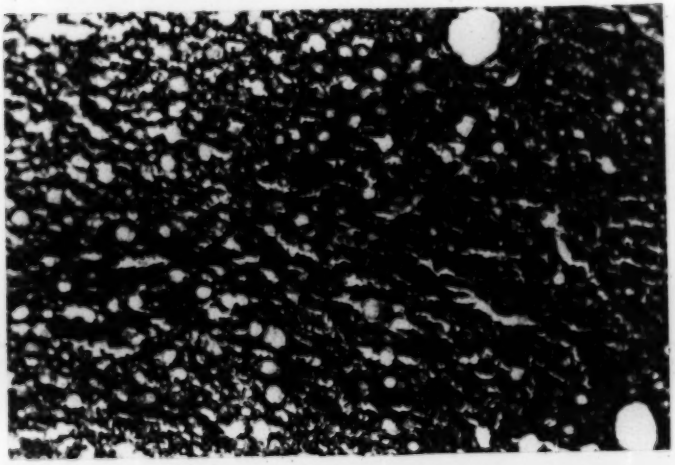
C. A focal area of mononuclear inflammatory cells in the white matter near the edge of the spinal cord.
X187.

UMI

A



B



C

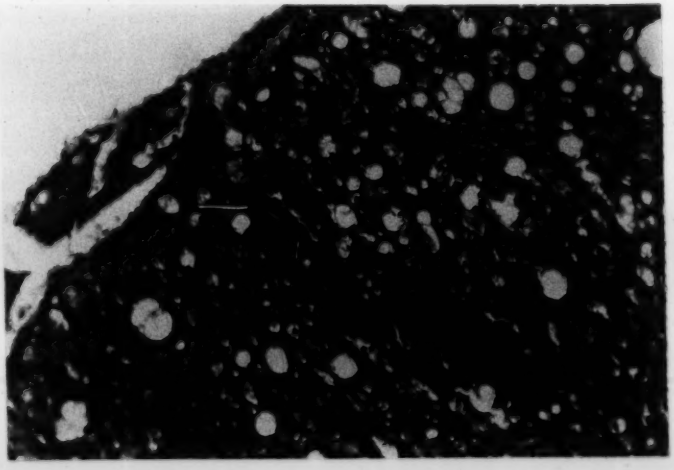


FIGURE 13.

EAE lesions in the CNS of a rat sensitized with 8,000 µg of whole guinea pig spinal cord in complete Freund's adjuvant. This animal was the adult offspring of a female sensitized on the 8th day of pregnancy with 43.2 µg of rat myelin basic proteins in incomplete Freund's adjuvant.

A. Three focal areas of mononuclear inflammatory cells in the white matter of the spinal cord.
X187.

B. Three focal areas of mononuclear inflammatory cells in the white matter of the cerebellum.
X198.

C. A focal area of mononuclear inflammatory cells in the white matter near the edge of the pons.
X364.

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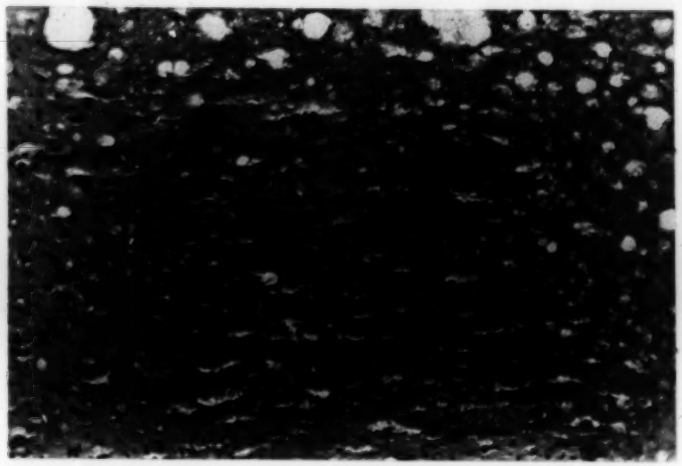
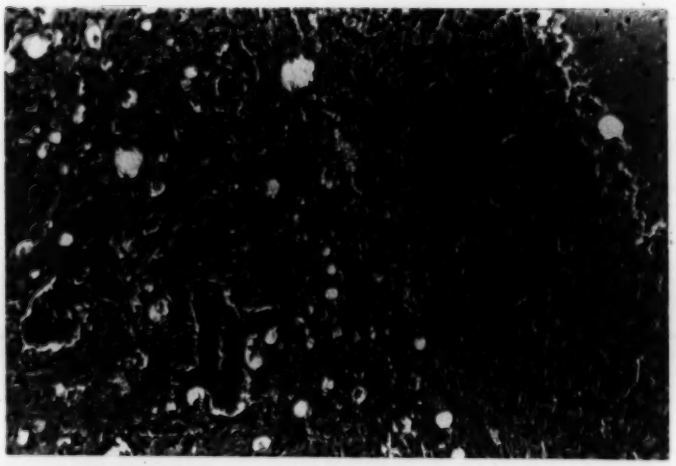
A



B



C

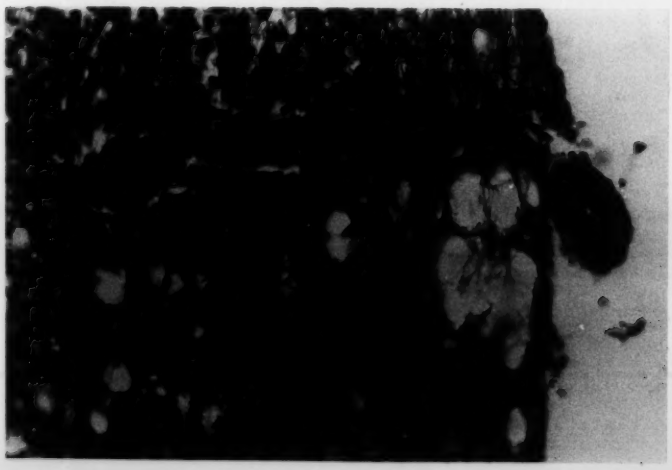


FIGURE 14.

EAE lesions in the CNS of rats sensitized with 10,000 µg of whole guinea pig spinal cord in complete Freund's adjuvant. These animals were the adult offspring of females sensitized on the 4th day of pregnancy with 43.2 µg of rat myelin basic proteins in incomplete Freund's adjuvant.

A. Focal and diffuse infiltrations of mononuclear inflammatory cells in the white and gray matter of the spinal cord.
X198.

B. Focal and diffuse infiltrations of mononuclear inflammatory cells in the white matter of the cerebellum.
X198.

C. Diffuse infiltrations of mononuclear inflammatory cells in the white matter of the pons.
X198.

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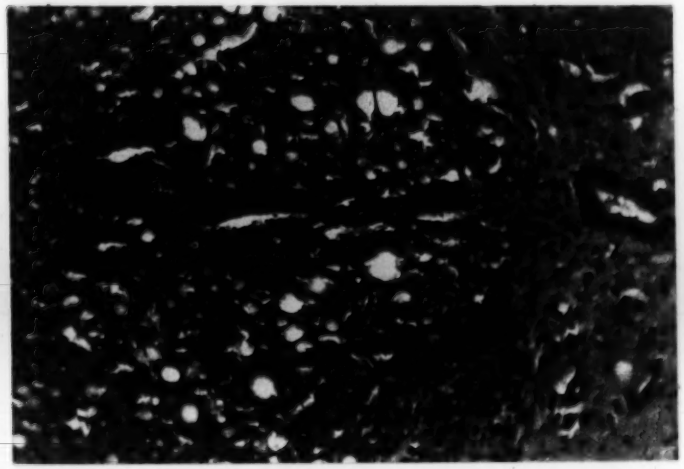
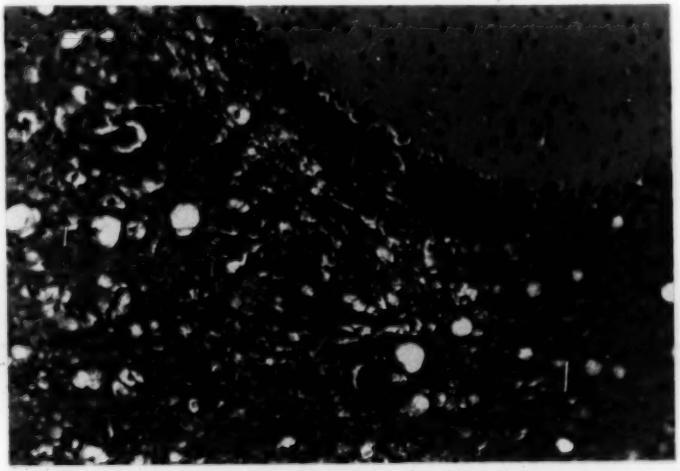


A



B

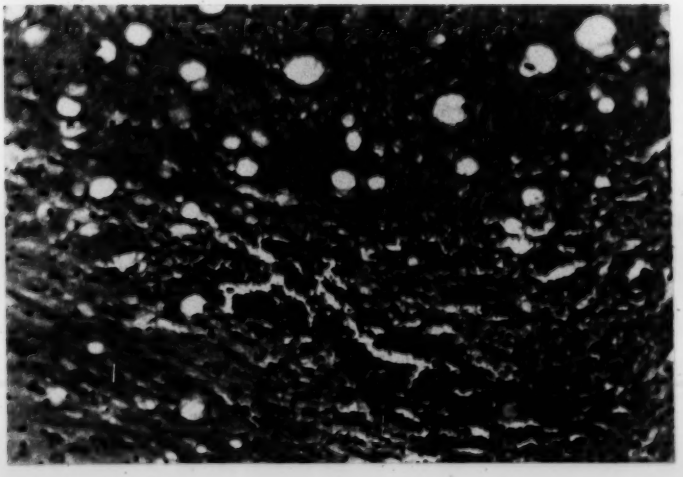


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FIGURE 15.

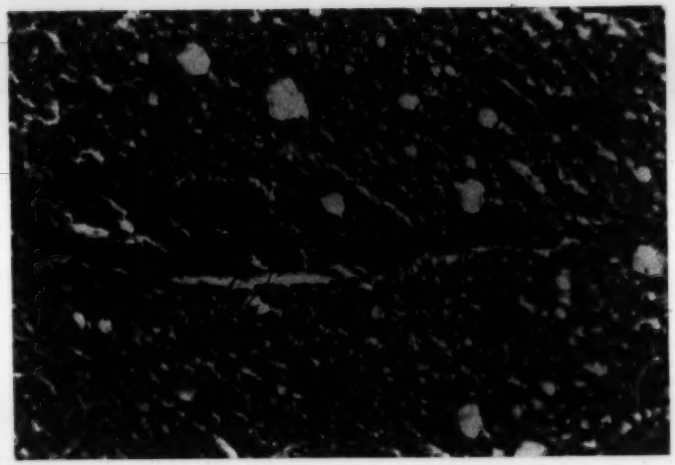
EAE lesions in the CNS of rats sensitized with 43.2 µg (A) and 259.2 µg (B) of rat myelin basic proteins in complete Freund's adjuvant. These animals were the adult offspring of females sensitized on the 8th day of pregnancy with 43.2 µg of rat myelin basic proteins in incomplete Freund's adjuvant.

A. Focal and diffuse infiltrations of mononuclear inflammatory cells in the white matter of the spinal cord.
X198.

B. A narrow focal area of mononuclear inflammatory cells in the white matter of the spinal cord.
X187.

UMI

A



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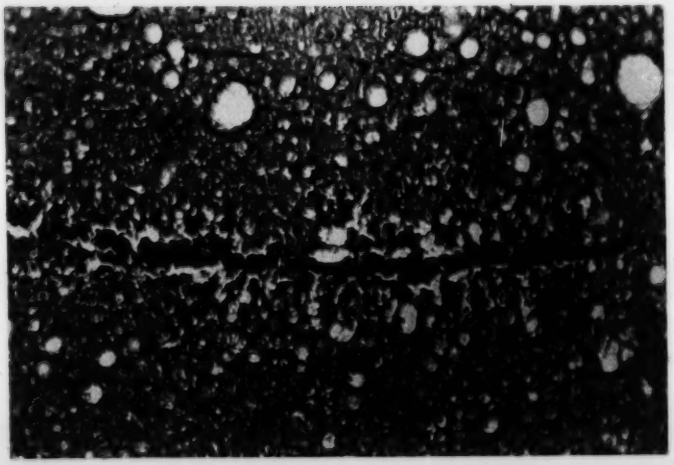

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FIGURE 16.

EAE lesions in the CNS of rats sensitized with 86.4 µg (A) and 129.6 µg (B) of rat myelin basic proteins in complete Freund's adjuvant. These animals were the adult offspring of females sensitized on the 4th day of pregnancy with 43.2 µg of rat myelin basic proteins in incomplete Freund's adjuvant.

A. Focal and diffuse infiltrations of mononuclear inflammatory cells in the white matter of the spinal cord. The infiltrations extend inwards to the edge of the gray matter.
X192.

B. Diffuse infiltrations of mononuclear inflammatory cells in the white matter of the spinal cord.
X198.

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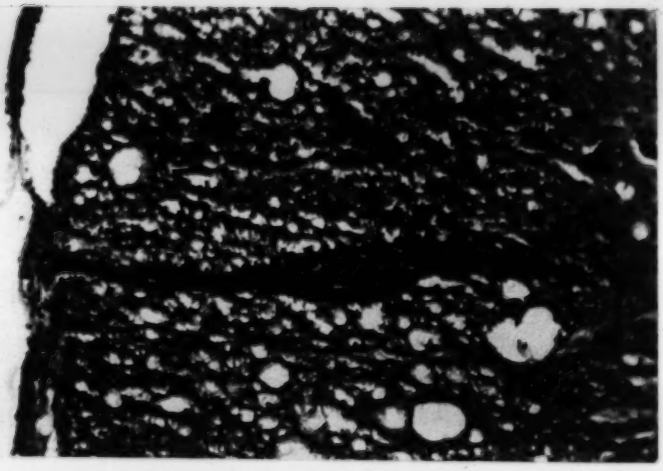
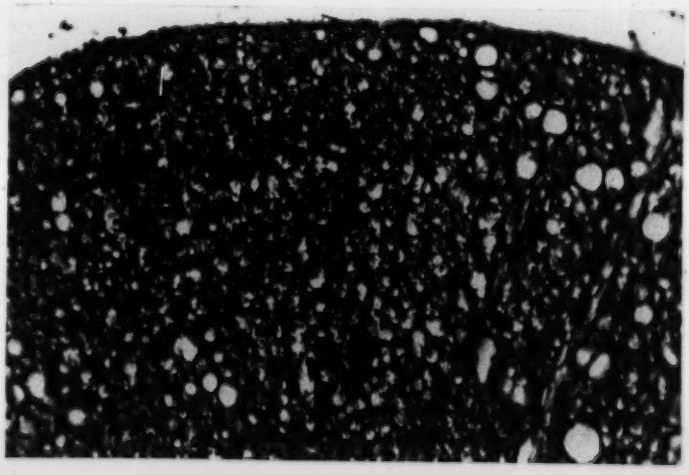
A



B



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